Glucocorticoid receptor activation by long acting steroids and its modification by inflammation

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TABLE OF CONTENTS

Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

Figures

CHAPTER 5

ABBREVIATIONS

ASMC: airway smooth muscle cell

BEC: bronchial epithelial cells

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

C/EBPα: CCAAT/enhancer binding protein alpha

CBP: CREB binding protein

CDK: cyclin-dependent kinase

cDNA: complementary deoxyribonucleic acid

cm: centimetre

$CO_{2:}$ carbon dioxide

COPD: chronic obstructive pulmonary disease

CREB: cAMP responsive element binding protein

DMSO: dimethyl sulfoxide

EDTA: ethylenediamine tetraacetic acid

FCS: fetal calf serum

FKBP: FK506 binding protein

GM-CSF: granulocyte/macrophage-colony stimulating factor

GR: glucocorticoid receptor

GRE: glucocorticoid-response elements

HAT: acetyl transferase

HDAC: histone deacetylase

HSP90: heat shock protein 90

IL: Interleukin

JNK: c-Jun N-terminal kinase

kDa/kD: kiloDalton

Kip1: cyclin-dependent kinase inhibitor 1

Abbreviations

MAPK:	mitogen-activated protein kinase
MEK:	MAPK kinase (MAP2K)
MEKK:	MAPK kinase kinase (MAP3K)
NF-*k*B:	nuclear factor-kappa B
P38:	p38 MAP kinase
PCR:	polymerase chain reaction
PCAF:	p300/CBP activating factor
PKB:	protein kinase B, Akt
RNAi:	RNA interference
SRC:	steroid receptor co-activator
STAT:	signal transducer and activator of transcription
TGF-β1:	transforming growth factor-beta1
TNF-α:	tumor necrosis factor-apha
℃:	Celsius
μg:	microgram
μl:	microlitre
μM:	micromolar

SUMMARY

summary

Glucocorticoids have an effective anti-inflammatory, anti-proliferative and immuno-modulatory activity. Therefore this class of drugs is used worldwide for the treatment of inflammatory diseases and to prevent rejection following organ transplantation. Inhaled glucocorticoids are the cornerstone treatment of asthma and advanced chronic obstructive pulmonary disease (COPD). The new long acting glucocorticoids mometasone and Ciclesonide have recently been introduced for the treatment of asthma.

The aim of these studies is to assess the kinetics and molecular pathways of glucocorticoid receptor (GR) activation and traffic in human lung fibroblasts and bronchial smooth muscle cells. Furthermore the effects of new long acting glucocorticoids on GR activation, complex formation with the transcription factor C/EBP and its isoforms proliferation will be evaluated.

Glucocorticoids enter the cytosol of cells by diffusion and bind to an intracellular specific receptor, the glucocorticoid receptor (GR), inducing a conformational change and leading thereby to its activation. The active GR translocates into the nucleus, where it binds to its cognate DNA recognition sequence, the glucocorticoid response element (GRE). Often the activation of the GR is associated with a parallel activation of the transcription factor C/EBP-α, followed by *de novo* synthesis of $p21^{(waf1/cip1)}$, which in turn is mediating the GR dependent inhibition of the cell cycle. Both, GR and C/EBP-α, are necessary to induce and activate $p21^{(waf1/cip1)}$ and to arrest the cell cycle at the transition from the G_1- to the S-phase.

Fibroblasts and bronchial smooth muscle cells are centrally involved in airway remodeling of patients with asthma and COPD. Therefore three cell types were chosen to assess the effect of the classical and new glucocorticoids on GR activation, cell proliferation and extracellular matrix deposition under different conditions. Four cell culture conditions were chosen to mimic different stages of inflammation: confluent cells without serum representing intact, not damaged tissue; confluent cells with 5% fetal calf serum to mimic early stages of inflammation; sub-confluent cells without serum to represent damaged tissue with no active inflammatory process; sub-confluent cells with 5% serum to represented tissue with active inflammation-induced lung tissue remodeling.

The results showed that cell density and inflammation alter the localization and function of the GR. In sub-confluent cells dexamethasone activated the nuclear accumulation and DNA binding of the GR persistently, while in confluent cells its activity declined. In sub-confluent

cells, GR interacted with a 42 kDa C/EBP-α isoprotein, which resulted in an up-regulation of $p21^{(Waf1/Cip1)}$ expression and suppression of proliferation. In confluent cells glucocorticoids induced $p27^{(Kip1)}$ expression via p38 MAP kinase and a 52 kDa C/EBP-β isoprotein. Furthermore, $p27^{(Kip1)}$ did not mediate the anti-proliferative effect of glucocorticoids, but simultaneous inhibition of $p21^{(Waf1/Cip1)}$ and $p27^{(Kip1)}$ unlocked contact inhibition in confluent cell cultures.

The studies further showed that in lung fibroblasts the long acting steroid mometasone in contrast to ciclesonide or the short acting steroids dexamethasone, budesonide or fluticasone did not induce tachyphylaxis and had a preferable effect on airway remodeling. Mometasone maintained the increased GR activity significantly over 24 hours, compared to that induced by dexamethasone which peaked at 6 hours and declined thereafter. Similarly drug specific effects were observed for the expression of C/EBP-α and $p21^{(Waf1/Cip1)}$. The anti-proliferative effect of mometasone was significantly stronger and long lasting compared to all other steroids. Similar anti-proliferative effects were observed in bronchial smooth muscle cells. Regarding airway remodeling we observed that mometasone, in contrast to all other steroids, did not further stimulate serum dependent synthesis and deposition of extracellular matrix and did not affect matrix metalloproteinase-2 and -9 expression or activity. Therefore this novel steroid will have a lower modulatory effect of tissue remodeling compared to the other steroids. Interestingly the long duration effects of the new glucocorticoid ciclesonide originated from the metabolism of the epithelial cells.

In conclusion, our findings suggest that the action of glucocorticoids differs under non-inflammatory and inflammatory conditions in human lung fibroblasts and bronchial smooth muscle cells. Different doses of inhaled steroids might therefore be applied in patient with asthma and COPD dependent on the current status of inflammation in order to reduce side effects. The long acting glucocorticoid mometasone might help to reduce overall steroid dosage and might thereby reduce the risk of tachyphylaxis, enhanced tissue remodeling and other typical steroid side effects. Ciclosonide is a new long acting steroid, but its action on mesenchymal cells depends on the function of epithelial cells.

These studies not only improve the understanding of the effect of different glucocorticoid on mesenchymal lung cells but might also help to find optional treatment doses.

CHAPTER 1

Introduction

Glucocorticoids, also known as glucocorticosteroids, cortcoisteroids or simply steroids are endogenously synthesized steroid hormones of the adrenal cortex and distributed by the blood circulation, regulating a variety of cell-, tissue- and organ-specific biological functions including immuno-modulation, metabolism, renal function, vascular leakage, cell growth, differentiation and death. Glucocorticoid synthesis is controlled by the hypothalamic–pituitary–adrenal (HPA) axis. The major steroid hormones produced by humans are cortisol (hydrocortisone) and corticosterone which regulates stress and inflammatory response, lipid, carbohydrate and protein metabolism. Mineralocorticoids such as aldosterone act via their specific receptor and regulate mainly the water and salt balance in the kidney. Andogens and estrogens are steroids that regulate sexual development and function. Most synthetic steroids are designed to modulate the function of the first group of steroids, the cortisones.

The glucocorticosteroid and its signaling pathway are among the most preserved mechanisms in evolution. Clinically, synthetic glucocorticoids represent a group of the most commonly prescribed drugs worldwide, and, are among the most potent anti-inflammatory drugs. They are also effectively used for their anti-inflammatory or immune-suppressive effects in asthma, dermatitis, rheumatoid arthritis, prevention of rejection in transplantation, and autoimmune diseases. Inhaled glucocorticoids have become the first line therapy in asthma. The molecular mechanisms how glucocorticoids suppress inflammation in asthma is based on their effect on gene transcriptions and signaling pathways (Barnes,1998;Barnes, 2006;Coghlan et al., 2003;Adcock et al., 2006a).

1.1 The Glucocorticoid Receptor (GR)

Glucocorticoids mediate their effect via their intracellular receptor, the glucocorticoid receptor (GR), which belongs to a family of intracellular steroid hormone receptors that act primarily as transcription factors controlling gene activity.

The cDNA of human glucocorticoid receptor (hGR), was first cloned in 1985 (Hollenberg et al., 1985), and later mapped to chromosome 5q31-32 (Theriault et al., 1989;Tsai and O'Malley, 1994). The GR protein possesses a modular structure consisting of three major domains—the N-terminal (NTD), DNA binding (DBD), and ligand binding (LBD). The GR gene has 10 exons spanning a 110 kb region. The 184 nucleotides of exon 1 represent solely the 5'-untranslated region. Exon 2 (1197 bp) encodes most of the receptor N-terminus,

including the constitutive AF1 transactivation domain. The two zinc-finger motifs involved in DNA-binding are separately encoded by exon 3 (167 bp) and exon 4 (117 bp). Five exons (exons 5, 6, 7, 8, 9) together make up the ligand-binding domain and ligand-dependent AF2 as well as the 3'-untranslated regions. The promoter of the GR lacks a TATA box for transcriptional activation and it also does not have a CCAAT motif in the 5'-flanking region. Instead, multiple GC boxes, activator protein-1 (AP-1), AP-2, Sp1, cAMP-responsive elements (CRE), Yin Yang1 (YY1), nuclear factor-kappa B (NF-κB) and several tissue-specific transcription factor-binding sites have been identified that regulates its expression. This information is consistent with the notion that GR is constitutively expressed in virtually every cell type, but with a tissue-specific pattern (Hach é et al., 1999;Necela and Cidlowski, 2004;Kumar and Thompson, 2005;Tsai and O'Malley, 1994;Hayashi et al., 2004a) (figure.1). Alternative splicing of pre-RNA generates two different isoforms of hGR: hGRα and hGRβ.

In addition, there exists a membrane specific GR (Bartholome et al., 2004; Jain et al., 2005). The classic hGRα, consists of 777 amino acids and is expressed by all cell types. The hGRβ is of smaller size generated by splicing of the last exon, resulting in a protein of 742 amino acids that diverges at its C terminus, and the final 15 amino acids of the C-terminal domain are unique to hGRβ. The hGRβ seems to act mainly as a dominant negative factor that blocks the function of hGRα as a transcription factor. The ratio of GRα:hGRβ expression is indeed critical to the glucocorticoid responsiveness of various cells. This ratio can be altered by changing the expression level of the hGRα, or the hGRβ, or of both receptors. Higher ratios of hGRα:hGRβ correlate with glucocorticoid sensitivity, while lower ratios correlate with glucocorticoid resistance (Lewis-Tuffin and Cidlowski, 2006).

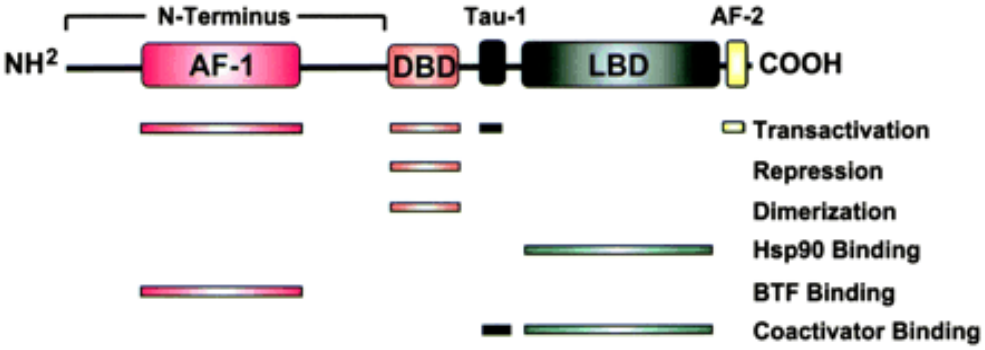

Figure 1. *A diagram of the molecular nature of the human glucocorticoid receptor. Adapted from (Necela and Cidlowski, 2004)*

1.2 GR signal transduction

The hGR is a 94 kDa protein, and the inactive hGR is located in the cytoplasm, where it is complexed with heat shock proteins (HSP) 70, HSP90, and the immunophylins p59, phosphoprotein p23 and FKBP51 (Hach é et al., 1999;Pratt et al., 1992;Pratt et al., 2006). The GR is activated by binding of a glucocorticoid inducing a structural transformation, disassembly of the multi-protein complex, followed by subsequent homodimerisation with a second GR molecule. The GR-dimer is then linked via FKBP51 for trafficking along microtubules which translocates the GR-complex into the nucleus (Hach é et al., 1999;Umland et al., 2002;Davies et al., 2002). In the nucleus, the dimerized GR binds into the major groove of the DNA through its central zinc finger DNA-binding domain, which recognizes distinct palindromic DNA sequences, termed glucocorticoid response elements (GREs). The GRE consensus sequence is 5'-TGTACAnnnTCTTGT-3'(where "n" represents any nucleotide) (Almawi and Melemedjian, 2002), which is usually located in promoters of glucocorticoid responsive genes. Binding of the GR to the GRE results in inducing conformational change within the receptor (Starr et al., 1996). The allosteric interaction promotes the recruitment of several co-activator complexes critical for remodeling of chromatin structure. However, many GR responsive genes do not have a GRE, but the GRE interferes with several other transcription factors and/or coactivators and thereby affects the transcription of this GRE-less genes. The binding of the GR complex to the GRE can either stimulate or silences the respective genes (Adcock and Lane, 2003). The copy number of the GRE differs among gene promoters as does their sequence in a promoter. In addition the distance of the GRE to other transcription factor binding sites is important for the effects of glucocorticoids on gene activity.

The GR interacts with transcription initiation factors such as the cAMP response element–binding protein (CREB), CREB-binding protein (CBP/p300) and the p300/CBP associated factor (p/CAF). These proteins contain intrinsic histone acetylase activity, which resulted in opening up to the chromatin structure of the coiled DNA, to allow binding of RNA polymerase II and initiates gene transcription. Several transcription factors interact with CBP, including CREB, NF-kB and AP-1 and these coactivators acetylate lysine residues in core histones to induce nucleosomal rearrangement of the DNA (Smirnov, 2002;Bruna et al., 2003;Jenkins et al., 2001;Deroo and Archer, 2001).

Other coactivator complexes such as steroid receptor coactivator-1, p/CIF, SWI/SNF, and GRIP1/TIF2/NcoA-1 (GRIP: glucocorticoid receptor interacting protein) further contribute to this chromatin-remodeling process (Smirnov, 2002;Bruna et al., 2003;Jenkins et al., 2001;Deroo and Archer, 2001). Nucleosomal rearrangement leads to promoter accessibility and the recruitment of the basal transcriptional machinery, including TATA box-binding protein (TBP), TBP-associated factors, and RNA polymerase II. The concerted assembly of these factors results in the stimulation of selective gene transcription. (Davies et al., 2002;Smirnov, 2002;Bruna et al., 2003;Jenkins et al., 2001;Deroo and Archer, 2001;Pelaia et al., 2003;Adcock et al., 2006a). An overview of this transcription complex and the role of the GR is shown in figure 2 (Pelaia et al., 2003).

1.3 GR signal pathway cross talking with other transcription factors

In addition to direct binding to GRE promoter elements, the GR also works through its association with other transcription factors. Thereby the GR can become involved in many transcription factor signalluing pathways and expand the GR function through cross talking with other signal pathways that makes the evaluation of such a signal network complicated. For example, in the mouse liver the presence of exogenous glucocorticoid modulates over 1,300 genes and here the GR binds to more than 300 promoters, but only the activity of 53 genes is functionally regulated upon the direct ligand-bound GR (Phuc et al., 2005).

The activated GR interacts with other transcription factors including C/EBP-α, C/EBP-β, Stat3/5, NF-κB, or AP-1(Rogatsky and Ivashkiv, 2006a;Bruna et al., 2003;Zhang et al., 1997;Floyd and Stephens, 2003;Aljada et al., 1999;Gotoh et al., 1997;Adcock et al., 1996a;Borger et al., 2002;Cha et al., 1998;Rüdiger et al., 1999a). This protein-protein interaction of the GR with other transcription factors does not need the translocation of the active GR into the nucleus and occurs in the cytosol (Wikström, 2003). Table 1 provides a summary of genes, which are controlled by the GR.

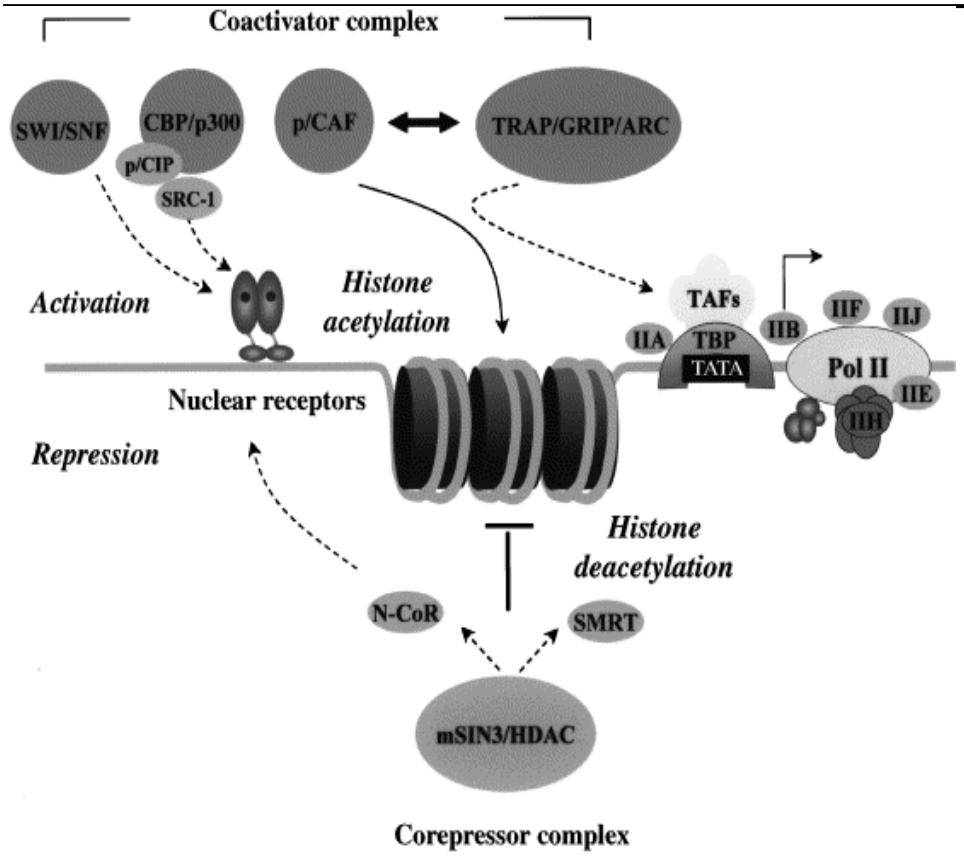

Figure 2. *Nuclear receptors can interact with coactivator complexes including CBP (CREB-binding protein)/p300 and p/CAF (p300/CBP associated factor) that possess histone acetyltransferase activities, p/CIP (p300/CBP co-integrator associated protein), SRC-1 (steroid receptor coactivator 1), and the SWI/SNF complex which possesses ATP-dependent chromatin remodeling activities. All these complexes may act in concert to relieve chromatin-mediated gene repression, with the TRAP (thyroid hormone receptor associated protein)/GRIP (glucocorticoid receptor interacting proteins)/ARC (activated recruited cofactor) complex functioning to recruit the core transcription machinery. The latter includes the TATA box-binding protein (TBP), the TBP associated factors (TAFs), the general transcription factors IIA, IIB, IIE, IIF, IIH, IIJ, and the enzyme RNA polymerase II (Pol II). Nuclear receptors can also interact with the corepressors N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor) thus leading to the recruitment of the mSIN3/HDAC (histone deacetylase) corepressor complex, possessing histone deacetylase functions. This corepressor complex can thereby inhibit gene transcription by counteracting the actions of the coactivator complexes containing histone acetyltransferase activities.*

1.3.1 NF-κB

NF-κB is assumed to be one of the pivotal transcription factors contributing to synthesis of pro-inflammatory factors in inflammatory diseases (Barnes and Adcock, 1997). NF-κB regulates several inducible genes, including nitric oxide synthase (iNOS), and the inducible form of cyclo-oxygenase (COX-2). It stimulates chemotactic cytokines (chemokines) such as interleukin (IL)-8 and eotaxin, and adhesion molecules such as ICAM-1 and VCAM-1 that

play a key role in inflammatory cell recruitment (Barnes and Adcock, 1998;Beato et al., 1996). Inactive form of NF-κB is located in cytoplasm and complexes with an inhibitory protein, IκB. Upon cell activation, IκB kinase-2 (IKK2) phosphorylates IκB, and after ubiquitination, the proteasome rapidly degrades IκB, releasing free form of NF-κB, NF-κB itself has two subunits, a p65 (or named Rel A) subunit and p50 subunit, both of which are members of the Rel family (Karin, 1999;Hart et al., 1998). Free NF-κB enters the nucleus as a p50-p65 heterodimer that binds to a κB recognition sites in the 5'-promoter (upstream) region of inflammatory genes, stimulating their synthesis. The p50-p65 heterodimer NF-κB can also bind to CBP or PCAF, this binding resulting in acetylation of lysines in core histone-4 and

Table：The effect of glucocorticoids on gene transcription, adapted from (Barnes, 2006;Hayashi et al., 2004b)

Increased transcription	**Decreased transcription**
Annexin 1 /lipocortin-1(phospholipase A_2 inhibitor) ß$_2$-Adrenergic receptor Secretory leukoprotease inhibitor(SLPI) CC10 (phospholipase A_2 inhibitor) IL-1 receptor antagonist IL-1 receptor type II (decoy receptor) IκB-α GILZ MKP-1(MAP Kinase phosphatase1) IL-10 (indirectly) CD163(scavenger receptor)	**Cytokines:** IL-1 to IL-6, IL-9, IL-11 to IL-13, IL-16 to IL-18, TNF-α, GM-CSF, SCF **Chemokines**: IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxins **Adhesion molecules**: ICAM-1, VCAM-1, E-selectin **Inflammatory enzymes**: iNOS, inducible COX-2, $cPLA_2$ **Inflammatory receptors**: Tachykinin NK_1 and NK_2 receptors, Bradykinin B_2 receptors **Peptides:** Endothelin-1

changes the structure of the DNA coil opening it up to give access to other transcription initiating proteins (Cayrol and Ducommun, 1998;Ito et al., 2000), it may need both

mechanisms to activate the transcription of a pro-inflammatory gene histone acetylation and promoter binding (Karin and Chang, 2001;Barnes and Adcock, 1995;Barnes, 1998;Karin and Chang, 2001) (see figure.2).

Glucocorticoids inhibit histone acetylase (HAT) activity directly and specifically recruit histone deacetylase (HDAC)-2, which reverses histone acetylation leading to the suppression of inflammatory genes (Adcock et al., 2004;Barnes and Adcock, 1995;Karin and Chang, 2001). Glucocorticoids inhibit NF-*κ*B activity also by induce IκB synthesis and thus antagonise NF-*κ*B activation (Auphan et al., 1995;Scheinman et al., 1995b;Scheinman et al., 1995a).

1.3.2 β_2- Adrenergic receptor

The human β_2-Adrenergic receptor (β_2-AR) gene is situated on the long arm of chromosome 5 and encodes for an intronless gene product of approximately 1200 base pairs, which is classically identified in cardiac and airway smooth muscle cells. β_2-AR is one of the G protein–coupled receptors, and has 7 trans-membrane spanning a-helices. The β_2-AR oscillates between activated and inactivated forms, thereby controlling its function. (Johnson, 2006). The coupling of the β_2-AR to adenylate cyclase occurs through a trimeric G protein, consisting of α-subunit (which stimulates adenylate cyclase) and βγ-subunits (which transduce other signals). Activation of the β_2-AR by β_2-agonists causes the α-subunit of the associated G protein to dissociate and couple with adenylate cyclase, leading to enhanced production of cAMP, a very important second signal messenger and also stimulates of PKA. PKA induces phosphorylation of other proteins and uncouples the G protein from the β_2-AR. The phosphorylated receptor, with its residual βγ-subunits, then couples to Gi which binds to β-arrestin, and then acts as an assembly scaffold for Src, SOS, and RAS, which in turn activates the MAPK pathway (Adcock et al., 2002;Johnson, 2006;Adcock et al., 1996b).

In primary human lung fibroblasts and smooth muscles, salbutamol and salmeterol induced the nuclear accumulation of the GR and enhanced GR-GRE binding in the absence of steroids. The translocation of the GR into the nucleus by β_2-agonists was less effective than by a steroid and was PKA dependent (Eickelberg et al., 1999). Long-acting β_2-agonists may affect GR nuclear localization priming of GR functions within the nucleus by modifying GR or GR-associated protein phosphorylation. Furthermore, PKA phosphorylates serine 276 of

the p65 subunite of NF-κB and thus enhance NF-κB-GR cross-talking (Haske et al., 1994). However, long term administration of β_2-agonists *in vivo* down-regulates the expression of β_2-AR, while glucocorticoid treatment increased the number of β_2-AR in the human lung (Davies and Lefkowitz, 1984;Davies and Lefkowitz, 1981). Treatment with β_2-agonists inhibits cell proliferation, and needs the activation of negative cell cycle regulator $p21^{(waf1/cip1)}$ gene (Rüdiger et al., 1999b).

In regard to the effect of β_2-agonists on GR activation it is important to note that cAMP activates the transcription of the GR promotor (Penuelas et al., 1998). cAMP activates protein kinase A (PKA), which phosphorylates a transcription factor CREB that binds to a cAMP response element (CRE) in the promoter region of certain genes. CREB is a member of large family of CRE binding proteins, including members of the ATF family. As described above CREB binds to CBP that acts as a co-activator molecule that binds to the TATA box and initiates transcription. CREB may be counteracted by another transcription factor called CRE modulator (CREM) that may block the effects of CREB on CRE.

CREB also appears to be important in the regulation of β_2-AR expression as it is activated by relatively high concentrations of β_2-agonists in the lung and may play a role in the down regulation of β_2-AR after chronic treatment with β_2-agonists. CREB has a negative effect on AP-1 and GR, and high concentrations of β_2-agonists inhibit the binding of GR to the GRE sequence. This may interfere with the anti-inflammatory effects of steroids and may account for the deleterious effects of high dose inhaled β_2-agonists in patients with asthma. The cAMP responsiveness can occur in the absence of CREB when C/EBP-α functionally substitutes for CREB. This finding suggests that the mechanism whereby C/EBP-α mediates constitutive transactivation is distinct from that whereby it mediates cAMP responsiveness (Penuelas et al., 1998).

1.3.3 The effect of the GR on p38 MAPK pathway

The intracellular signal transducing protein p38 MAPK is activated by inflammatory stress through activation of MAPK kinase (MKK)-3 and -6. Activated p38 MAPK phosphorylates MAPK-activated protein kinase (MAPKAPK)-2, which stabilises mRNA encoding several inflammatory proteins, such as tumor necrosis factor (TNF)-α, IL-1β, IL-6,

IL-8, GM-CSF and COX-2 (Smoak and Cidlowski, 2004;Page and Hershenson, 2000;Szatmary et al., 2004). All these mRNA are characterized by adenine–uracil-rich elements (AREs) in the 3'-untranslated region, which makes the mRNA unstable and rapidly degraded. ARE-binding proteins (AREBPs) stabilize these proteins and may be activated by MAPKAPK-2(Page and Hershenson, 2000;Szatmary et al., 2004).

Glucocorticoids induce the expression of MAPK phosphatase (MKP)-1, which inhibits p38 MAPK and, thus, prevents the stabilization of multiple inflammatory proteins (Pelaia et al., 2003;Barnes, 2006). The pattern of MAPK expression, activation and pharmacological modulation differs among the various cell types and tissues when exposed to endogenous or synthetic glucocorticoids, and the ability of glucocorticoids to block MAPK phosphorylation is at least in part cell-type or stimulus-specific. Glucocorticoid-induced MAPK inhibition seems to be mediated by an increased expression, as well as by a decreased proteolytic degradation, of the MAP kinase phosphatase-1 (MKP-1) (Kassel et al., 2001).

1.3.4 TGF-β signal

The transforming growth factor-β (TGF-β) and the glucocorticoid signaling pathways interact both positively and negatively, thereby regulating physiological and pathologic processes The promoter region of the human TGF-β1 gene revealed a consensus GRE (5'-AGAACA) located from (-1081) to (-1086) base pairs (Parrelli et al., 1998). GR regulated TGF-β transcriptional activation involved both Smad3 and Smad4 C-terminal activation domains. GR interacts with Smad3 both *in vitro* and *in vivo* (Song et al., 1999;Chou et al., 2003). The molecular mechanism of TGF-β to exert its effect involves the activation of Smad proteins that control gene transcription directly or by the cooperation with other transcription factors, TGF-β has been to found to interact with the GR and thereby enhanced the glucocorticoid response of the mouse mammary tumor virus promoter (Aurrekoetxea-Hernandez and Buetti, 2000;Aurrekoetxea-Hernandez and Buetti, 2004). This effect may be explained by the observation that TGF-β increased glucocorticoid binding and signaling through a Smad 2/3- and AP-1-mediated mechanism (Peltier et al., 2003).

The interaction of the GR with TGF-β can occur on several levels and may include a feedback mechanism. The LBD of GR, but not the DNA-binding domain or the N-terminal activation domain, is required for GR-mediated transrepression of TGF-β transactivation (Li et al., 2003). However, TGF-β can also antagonize the growth inhibitory properties of GR by

blocking GR transactivion of various promoters through a mechanism involving transcriptional repression by DNA-bound AP-1 (Periyasamy and Sanchez, 2002).

Furthermore, TGF-β controls GR gene transcription through Smad2/3 by different mechanisms. First, Smad2/3 might interact with a putative Smad binding element (CAGACA) in the GR gene promoter. Second multimeric Smad-AP-1 complexes might interact with a single AP-1 site in the GR gene promoter. Third, Smad2/3 might also affect the transcription of genes encoding proteins that control indirectly GR gene expression, and TGF-β1 might increase the expression of the HSP 90, which is bound to the inactive GR and shapes the hormone binding domain into a ligand binding conformation (Bellocq et al., 1999;Aurrekoetxea-Hernandez and Buetti, 2004).

1.4 Cell cycle control and p21$^{(WAF1/CIP1)}$

Cyclins are the regulatory subunits of the holoenzyme cyclin dependent kinase (CDK) complexes, which control the progression through cell cycle checkpoints by phosphorylating and inactivating target substrates. The Cyclin family is divided into two main classes:

1. G_1-phase cyclins include cyclin C, D1-3, and E, and their accumulation is rate-limiting for progression from the G_{1-} to S-phase.

2. G_2-phase cyclins, which include cyclin A and cyclin B, are involved in the control of G_2/M-phase transition and control mitosis.

In different phases of the cell cycle, CDK4/6 bind to cyclin D, CDK2 binds to cyclins E or A and CDK1 binds to cyclins A or B (Martin et al., 2005b;Ortega et al., 2002b). The cyclins bind to and activate the CDKs, which leads to the phosphorylation of the tumor suppressor protein, pRb and thus to the inhibition pRB. pRb the progress from the G_1- to S-phase, at least in part by repressing the activity of the E2F transcription factors known to promote cell proliferation. Both the D-type cyclins and their partner kinases CDK4/6 activity is regulated at multiple levels including negative control by two families of CDK inhibitors. While members of the INK4 family (p16INK4A, p15INK4B, p18INK4C, p19INK4D) interact specifically with CDK4 and CDK6, the CIP/KIP inhibitors p21$^{(waf1/cip1)}$, p27KIP1 and p57KIP2 inhibit a broader spectrum of CDK (Golias et al., 2004). The interaction between

p16INK4A, cyclin D/CDK, and pRb/E2F together constitute a functional unit collectively known as the 'pRb pathway'. pRb is subject to different sets of multiple phosphorylations by cyclinD: CDK4/6 and cyclinE: CDK2. The E2F binding of p107, p130 and pRB, all is also inhibited by phosphorylation. Phosphorylation of E2F and/or DP by Cyclin A: CDK2 inhibits the E2F-DP interaction, releasing a free E2F and thus turn on transcription (Genovese et al., 2006).

The cyclin-dependent kinase inhibitor $p21^{(waf1/cip1)}$ is a major player in cell cycle control and it is mainly regulated at the transcriptional level. Which belongs to the Cip/Kip family of cdk inhibitors, it mainly inhibits the activity of cyclin/cdk2 complexes and negatively modulates cell cycle progression (Waga et al., 1994;Harper et al., 1993;Martin et al., 2005a;Ortega et al., 2002a). In addition, $p21^{(waf1/cip1)}$ can bind to proliferating cell nuclear antigen (PCNA) thereby blocking DNA synthesis. A large varity of factors are known to activate $p21^{(waf1/cip1)}$ including: p53, SP-1/SP-3, Smads, AP-2, STAT, E2F-1/E2F-3, CEBP-α and -β (Cram et al., 1998;Barnes, 2006), Glucocorticoids achieve their anti-proliferative function by interaction with theses factors. GR can form a complex with CEBP-α, and induces the translocation of the complex into the nucleus, where the complex through protein interactions of CEBP-α with $p21^{(waf1/cip1)}$ inhibits CDK2, and this mechanism is independent of transcriptional activity (Harris et al., 2001). CEBP-α and the GR also work by binding to the transcriptional responsive elements in the promoter of $p21^{(waf1/cip1)}$, inducing its transcription and thereby mediating cell cycle arrest (Ramos et al., 1996;Rüdiger et al., 2002;Gotoh et al., 1997;Cha et al., 1998). In addition, $p21^{(waf1/cip1)}$ also exerts its functions by directly blocking the ability of PCNA to activate DNA polymerase-δ, the principal replicative DNA polymerase, thus inhibit DNA replication (Waga et al., 1994). Previously, in our laboratory it was shown that GR activation by glucocorticoids and β_2-agonists led to the subsequent activation of $p21^{(waf1/cip1)}$ thereby inhibiting the proliferation of human lung fibroblasts, pulmonary vascular and bronchial smooth muscle cells (Eickelberg et al., 1999;Roth et al., 2000;Roth et al., 2002;Roth et al., 2004). Among the other transcription factors that interact with the $p21^{(waf1/cip1)}$ gene promoter, STAT is very important (Almawi et al., 1996;Almawi and Melemedjian, 2002;Rogatsky and Ivashkiv, 2006b).

1.5 Scope of this Thesis:

The use of synthetic glucocorticoids in asthma and COPD therapy is widespread due to their powerful anti-inflammatory, anti-proliferative and immuno-modulatory activity. However,

long-term use of these drugs can result in severe side effects. Long time inhaled at high doseage impaired growth of children, decreased bone mineral density, induced skin thinning with the consequence of bruising, and cataract development (Rosen and Miner, 2005). The search for novel glucocorticoids that have reduced side effects is being driven by the identification of new mechanisms of action of glucocorticoids and the GR. Understanding of the mechanism that regulates a specific gene by the glucocorticoid-signaling pathway may lead to more specifically targets for therapy, help to prevent unwanted side effects and help to provide some practical implications for the clinical use of glucocorticoids

Most side effects of glucocorticoids appear to be due to GR DNA binding and through the regulation of gene activity. Whereas the anti-inflammatory effect of this class of drugs is now adays assumed to be regulated pre-dominantly by the inhibition of inflammatory gene expression by interference of the GR with NF-kB activity and other pro-inflammatory transcription factors, therefore through a non-direct DNA binding mechanism which may be mediated via inhibition of HAT activity and HDAC recruitment (Smoak and Cidlowski, 2004;Adcock et al., 2004;Adcock et al., 2006a;Miner et al., 2005). This new aspect of GR action has led to a search for novel corticosteroids that selectively trans-repress gene activity without significant trans-activation or cis-repression, thus reducing the potential risk of side effects.

The increased understanding of transcription factors that are associated with the GR has given new insights into the pathophysiology of inflammatory disease such as asthma, and opened an opportunity for the development of new anti-asthma treatments. The steroids used in asthma therapy today, such as fluticasone propionate and budesonide, appear to have more potent trans-repression than trans-activation effects, which may account for their selection as potent anti-inflammatory agents (Jaffuel et al., 2000).

One of the most important implications of research on transcription factors is that of multiple and complex interactions between these proteins are possible and this leads to the so called cross talk between different signal transduction pathways. Transcription factors and their inhibitors or activators, maybe also the potential drug target (Roth and Black, 2006;Adcock and Caramori, 2004;Adcock et al., 2006b). However, when the targeted transcription factors are not cell- or tissue-specific their long-term general suppression or over-expression may

potentially cause severe side effects as well. It is well known that prolonged and continuous treatment of patients with corticosteroids leads to unwanted side effects, such as osteoporosis. A delivery system of transcription factor inhibitors and/or activators to a specific cell type would mean a tremendous improvement for drug targeting (Roth and Black, 2006).

More developments in the molecular mechanism of GR signal transduction provided theory supports for clinically therapy, combination of drugs that act on different transcript factors or pathways that may work together co-operatively. In asthma, glucocorticoids with β2-agonist treatment, the synergistic interaction so effective than either drug alone (Roth et al., 2002). In this study, it was attempted to describe the GR distribution and activation by different long acting steroids in inflammation conditions.

AIM OF THE THESIS

This thesis aims to assess the kinetics of the GR activation and traffic in human lung cells under different conditions of growth and inflammation. Furthermore the effect of the new long acting glucocorticoids mometasone and ciclesonide on GR activation, complex formation and cell growth will be evaluated.

Specific aims of the thesis will address the following topics:

–determination of the distribution and activation of the GR under different cell cultures conditions including cell density and inflammation.

–Characteration of the conditions of the GR with the anti-proliferative transcriptor factor C/EBP and its isoforms under different cell culture conditions.

–Comparison of the expression and activation of the GR the new long acting glucocorticoids mometasone and Ciclesonide with classical steroids including assessment of subsequent anti-proliferative pathways.

–Analysis of the effects of mometasone on extracellular matrix production and regulation by metalloproteinase.

–Evaluation of the mechanisms of activation and metabolism of Ciclesonide.

CHAPTER 2

Cell differentiation modifies the composition and function of the glucocorticoid receptor - C/EBP complex

When Glucocorticoids passively enter into the cytosol of the cells, it binds to GR and format a complex to enter the nucleus and then binding to GRE to control the cell activity. In our previous works, we found that GR can form a complex with C/EBP-α, which is essential to stimulate $p21^{waf1/cip1}$ expression thus the GR mediates its anti-proliferative effect in most human cell types.

Inflammation of the lung is characterized by vessel leakage and infiltration of serum into the surrounding tissue, thereby also exposing mesenchymal cells in undamaged tissue areas to serum containing pro-inflammatory factors. In this study low cell density and serum was used to mimic the condition of inflammation in which fibroblasts have to fill wounds to reconstitute damaged tissue. In this part of the studies I investigated the effect of cell density and the presence of serum on the activation and cell compartmental translocation of the GR in response to dexamethasone in cultures of primary human lung fibroblasts. I further investigated the role of the GR and C/EBP-α and –β on expression and complex formation in regard to their effect on two major anti-proliferative proteins $p21^{(Waf1/Cip1}$) and $p27^{(Kip)}$, and their activation by dexamethasone.

2.1 OBJECTIVES:

1. Is the GR distribution different at different cell culture conditons?
2. Does the serum affect the GR distribution and activation?
3. Does serum affect dexametasone-induced GR distribution and activity?
4. Does the cell density and cell differentiation affect the GR interaction with C/EBP isoforms and subsequently the action of p21/p27?

2.2 Cell differentiation modifies the composition and function of the glucocorticoid receptor - C/EBP complex

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Running title: Specific glucocorticoids receptor C/EBP complexes

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2.2.1 Abstract

The glucocorticoid receptor is a major control factor for cell proliferation, cell differentiation and inflammation. Our knowledge about the glucocorticoid receptor is focused on its function as a transcription regulator. However, cells are not always responding to steroids in the same way or develop tachyphylaxis or resistance. The mechanism underlying such a modified steroid response is not well understood and may depend on the microenvironment of the cells or on the stage of their differentiation. Therefore we studied the effect of cell density and inflammatory conditions on the expression, compartmentalisation, activation, and the anti-proliferative function of the glucocorticoid receptor in primary human lung fibroblast cultures. In sub-confluent cells the glucocorticoid receptor was located peri-nuclear, while in confluent cells it was ubiquitously expressed. Serum stimulation up-regulated the level of glucocorticoid receptor mRNA and protein under all conditions. In sub-confluent cells dexamethasone activated the nuclear accumulation and DNA binding of the glucocorticoid receptor persistently, while in confluent cells its activity declined after 6 hours. In sub-confluent cells, but not in confluent cells, the glucocorticoid receptor interacted with a 42 kDa, but not the 30 kDa C/EBP-α isoprotein, which resulted in an up-regulation of $p21^{(Waf1/Cip1)}$ expression and suppression of proliferation. In confluent cells glucocorticoids induced $p27^{(Kip1)}$ expression via p38 MAP kinase and a 52 kDa C/EBP-β isoprotein. However, $p27^{(Kip1)}$ did not mediate the anti-proliferative effect of glucocorticoids, but simultaneous inhibition of $p21^{(Waf1/Cip1)}$ and $p27^{(Kip1)}$ unlocked contact inhibition in confluent cell. Our results indicate that cell density and inflammation alter the localisation and function of the glucocorticoid receptor. (250)

Keywords: glucocorticoid receptor, primary human lung fibroblasts, proliferation control, tissue remodeling

2.2.2 Introduction

Glucocorticoids are expressed in virtually every known cell type and controls cell differentiation, organ development, and function. Glucocorticoids also regulate immune response, fat metabolism, renal function, and vascular leakage in most organisms (1, 2). Glucocorticoids bind to the inactive glucocorticoid receptor (GR) in the cytosol and induce the restructuring of the GR-multi protein complex. Two active GR molecules form a dimer that couples to FKBP52 and dynein and which mediate the transported into the nucleus (3, 4). In the nucleus the GR-dimer binds to a DNA sequence assigned as the glucocorticoid response element (GRE) and can either stimulate or silence genes (1, 5).

Interestingly, treatment with the steroids significantly down-regulated GR expression which suggests a self limiting control mechanism and may result in tachyphylaxis in long term steroid treatment (6-8). Beside its action as a transcription factor the GR mediates its effect also by binding to and modifying the function other transcription factors including C/EBP-α, -β, Stat1/5, NF_KB, or AP-1 (9-14). Specifically of interest for proliferation control are the complexes formed by the GR with the transcription factors C/EBP-α or –β as they are essential to initiate the expression of $p21^{(Waf1/Cip1)}$ and thereby mediate the anti-proliferative effect of steroids (10, 15, 16). Fibroblast proliferation is inhibited by glucocorticoids also via the activation of $p21^{(Waf1/Cip1)}$ which requires C/EBP-α activity (15, 17, 18). However, glucocorticoids do not always block proliferation in fibroblasts and the reason for this is unknown (19-21). The anti-proliferative efficacy of glucocorticoid may vary with the mitogenic stimulus (18), might be a species specific effect (20), or be affected by the organ of origin of the fibroblasts, or the underlying disease (19, 21). We described earlier that a cell type specific lack of C/EBP-α in bronchial smooth muscle cells of asthma patients is responsible for the enhanced proliferative capacity of this cells and possibly leads to airway muscle cell hyperplasia in asthma (22-24). In addition to airway smooth muscle hyperplasia the airway wall of asthma and COPD patients is also characterised by increased fibroblast proliferation and extracellular matrix deposition in the lamina propria (2, 25). The effect of steroids on these pathologies is controversial discussed and their effect may depend on drug dosage, length of therapy, and severity of the disease (2, 26). We have recently demonstrated that the effect of steroids on extracellular matrix and collagen deposition is modified by the

presence of serum in human lung fibroblasts. In resting cells or in the presence of TGF-β_1 steroids down-regulated the deposition of extracellular matrix, while in the presence of serum (5%) steroids further increased extracellular matrix deposition (26).

In regard to cell proliferation control it is important to note that cell density and the presence of mitogen modified the expression and function of $p21^{(Waf1/Cip1)}$ in fibroblast (27, 28). Furthermore, translocation of non-activated $p21^{(Waf1/Cip1)}$ into the nucleus of pancreatic fibroblasts was associated with differentiation into myo-fibroblasts which expressed muscle cells actin and were more susceptible to apoptotic signals (29). In an animal model for adipocyte differentiation cell density and serum affected the function of dexamethasone and the expression of C/EBP-α and –β (30). Taking into account that at least active C/EBP-α is required for the anti-proliferative effect of glucocorticoids we hypothesized that cell density, as well as mitogen, and cell differentiation change the function of the GR. Furthermore, glucocorticoids also activate a second cell cycle control protein, $p27^{(Kip)}$, but its role in the drug's effect is unclear and may be cell type or species specific (15, 31).

Therefore, we investigated the effect of cell density and the presence of serum on the activation and translocation of the GR in response to dexamethasone in cultures of primary human lung fibroblasts. We further investigated the role of the GR and C/EBP-α and –β on expression of two major anti-proliferative proteins $p21^{(Waf1/Cip1)}$) and $p27^{(Kip)}$, and their activation by dexamethasone.

2.2.3 MATERIAL AND METHODS

Chemicals: All chemicals were from Calbiochem (La Jolla, USA) if not otherwise notified. Complete protease inhibitor are: Roche Diagnostics (Basel, Switzerland), gradient poly-acryl amid gels (PAGE): Biorad (Hercules, USA). Minimal essential medium (MEM), RPMI 1640, phosphate buffered saline (PBS w/o Ca^{2+} and Mg^{2+}): Cambrex Bio Science Verviers (Verviers, Belgium), PVDF-membranes: Millipore (Bedford, USA), Ponceau: Sigma (Buchs, Switzerland), enhanced chemiluminescence (ECL): Pierce (Rockford, IL, USA) and X-ray films: Kodak (Eastman Kodak, Rochester, USA). Antibodies and small inhibitory (si) RNAs: Santa Cruz Biotechnology (Santa Cruz, USA). Cy3-labelled goat anti rabbit-IgG: Jackson ImmunoResearch Laboratories, USA. GRE oligonucleotides: Affinity Bioreagents Inc

(Golden, USA), all other DNA oligonucleotides MWG-Biotech GmbH (Ebersberg, Germany).

Fibroblast culture: Primary fibroblasts were established from lung tissue samples after written consent and approval by the ethical committee (University Hospital Basel). Fibroblasts were grown in RPM1-1640 supplemented with 10 % fetal calf serum (FCS) and 1 % MEM vitamins. All experiments were performed between cell passage two - six. Fibroblasts were seeded (1 x 10^4 cells/cm^2) and either used directly (subconfluent), or grown to 100% confluence + 2 days. Prior to experiments all cells were serum starved in 0.1 % FCS for 24 hours. For siRNA was used at a final concentration of 1-10 nMol for 24 hours prior to experiments. No specific transfection medium was used as fibroblasts readily absorbed siRNA within 24 hours, as confirmed by immuno-blotting.

Protein extraction, electrophoresis, co-immuno precipitation, and immuno-blotting: Cytosolic and nuclear protein fractions were isolated as described earlier and total protein concentration was determined by Bradfords' method (12, 17). Protein (10 μg) were dissolved in Laemmli buffer, denatured (95^{o}C, 5 min), chilled on ice (5 min), centrifuged (13'000 x g, 50 sec.), and applied to electrophoresis (4-15% SDS-PAGE). Proteins were transferred onto a PVDF membrane by semi-dry electro-transfer, which was confirmed by Ponceaus' staining. Membranes were washed 3 times with PBS, blocked with 5% skimmed milk in PBS (4^{o}C, overnight) and incubated with one of the primary antibodies (anti-GR antibody: 0.2 ng/ml, sc-1003; $p21^{(waf1/Cip1)}$: 0.2 ng/ml, sc-817; C/EBP-α: 0.2 ng/ml, sc-61; $p27^{(Kip)}$: 0.5 ng/ml, sc-1027). Unbound antibodies were washed off before membranes were incubated with horse radish-labeled species specific secondary antibodies for 1 hour at room temperature. After washing (3 x 15 min.) with blocking buffer and signals were detected by ECL substrate and documented on X-ray film (12, 17). Co-immuno precipitation for GR-C/EBP complexes was performed with the same antibodies used for immuno-blotting and followed the protocol described earlier (12).

Electrophoretic mobility shift assay (EMSA): EMSA was performed using a [^{32}P]-labelled GRE oligo-nucleotide (sc-2545) as described earlier (22, 26). Specificity of the GRE-protein complex was characterised by pre-incubating protein extracts with 50 fold excess of unlabeled GRE.

Immunochemistry and confocal microscopy: Fibroblasts were grown on cover slips (VWR International AG, Switzerland) treated and then fixed in methanol/acetic acid (3:1 vol:vol, 15 min., room temperature). Blocking (PBS, 5% donkey serum, 0.3% Triton-X-100, overnight, room temperature). For immuno-fluorescence slides were incubated with a primary Cy3-labelled goat anti rabbit-IgG antibody (blocking buffer, 1 hour, 4 ℃), followed by two washes with PBS and once with distilled water (5 min), then embedded in Fluorsave®. Images taken by a Zeiss LSM510 confocal microscope (Carl Zeiss AG, Jena, Germany) and analyzed using ImageJ v. 1.33.

Reverse transcriptase–polymerase chain reaction (RT-PCR): RNeasy® Mini kit (Qiagen, Basel, Switzerland) was used to extract total RNA which (1 μg) was transcribed into cDNA using murine leukemia virus reverse transcriptase (37 ℃, 60 min.) (Promega, Madison, US). PCR was performed for GR: forward 5'-CACCCTCACTGGCTGTCGCTTCTC-3', reverse 5'-TGACAAACGAAAGAGGAG ACCGCC-3', 23 cycles: denaturation: (98 ℃, 20 sec), annealing (58 ℃, 22 sec) and extension (72 ℃, 30 sec); p21 (primer set, R&D Systems Inc, Minneaplois, USA), 30 cycles: denaturation: (94 ℃, 45 sec), annealing (55 ℃, 45 sec) and extension (72 ℃, 45 sec);

β2-microglobulin (β2-M): forward 5'-CTCGCGCTACTCTCTCTCTTTCT-3', reverse 5'-TTAAGTGGGATCGAGACATGTAAGC-3', 23 cycles: denaturation (98°C, 30 sec), annealing (60 ℃, 60 sec) and extension (72 ℃, 60 sec). PCR products were size fractionated by electrophoresis in a 1 % agarose gel and products were visualized by ethidium bromide (12, 17).

Active and inactive GR by ELISA: GR GRE binding was assessed using an ELISA kit (Orgenium, Finland) distinguishing total from active GR as described by the distributor. GR activation was determined in the same protein extracts used for EMSA and immuno-blotting

Statistics: Results of protein activation kinetics were compared by paired ANOVA. The effect of signal protein inhibitors and siRNAs were compared by paired two-tailed student's t-test. The null-hypothesis was equality of response between groups. Results were considered to be significantly different when the probability (p) was < 0.05.

2.2.4 RESULTS

Cell density and serum modifies the cell compartmental distribution of the GR

Cell compartmental distribution of the GR was determined by three independent methods, confocal microscopy, immuno-blotting, and GRE-ELSIA. Serum (10%) did not significantly increase the cell compartment specific expression of the GR in sub-confluent fibroblasts within 24 hours, and the GR was located in the cytosol in a peri-nuclear location (Fig. 1A).

In contrast, in confluent serum starved cells the GR could be detected in the cytosol and the nucleus and was homogenously distributed and was significantly up-regulated by serum (10%) in both cell compartments as early as 3 hours (Fig. 1A). The serum stimulated increase of GR protein expression in confluent fibroblasts was preceded by *de novo* mRNA synthesis (Fig. 1B). Only in confluent cells the GR mRNA signal began to increase significantly at 0.5 hours peaking at 3 hours and declining thereafter (Fig. 1B). GR mRNA signals were suppressed by actinomycin D (10 μM), but not cycloheximide (10 μM) confirming mRNA *de novo* synthesis rather than accumulation (data not shown).

Activation of the GR by steroids is prolonged in sub-confluent cells

When sub-confluent fibroblasts were treated with dexamethasone (10^{-8} to 10^{-6} M) the GR was dose-dependently activated and translocated completely into the nucleus within 3 hours (immuno-blotting analysis). The level of the nuclear GR increased significantly within 0.5 hours compared to start levels ($p < 0.001$) and remained at a high level over 24 hours with no significant difference of the kinetic comparing serum starved cells to those serum (10%) stimulated cells (Fig.2A). In confluent fibroblasts the translocation of the GR into the nucleus was slower compared to sub-confluent cells, peaked at 1 hour, and declined thereafter to basal levels within 24 hours (Fig. 2A). Similar results were obtained using confocal microscopy and immuno-blotting. The functional activation of the nuclear GR was confirmed by GRE specific EMSA and ELISA, both methods showed that the increase of the GR in the nucleus was paralleled by binding of the GR to a synthetic GRE oligo-nucleotide and it confirmed the decline of GR activity in the presence of serum within 24 hour (Fig. 2B).

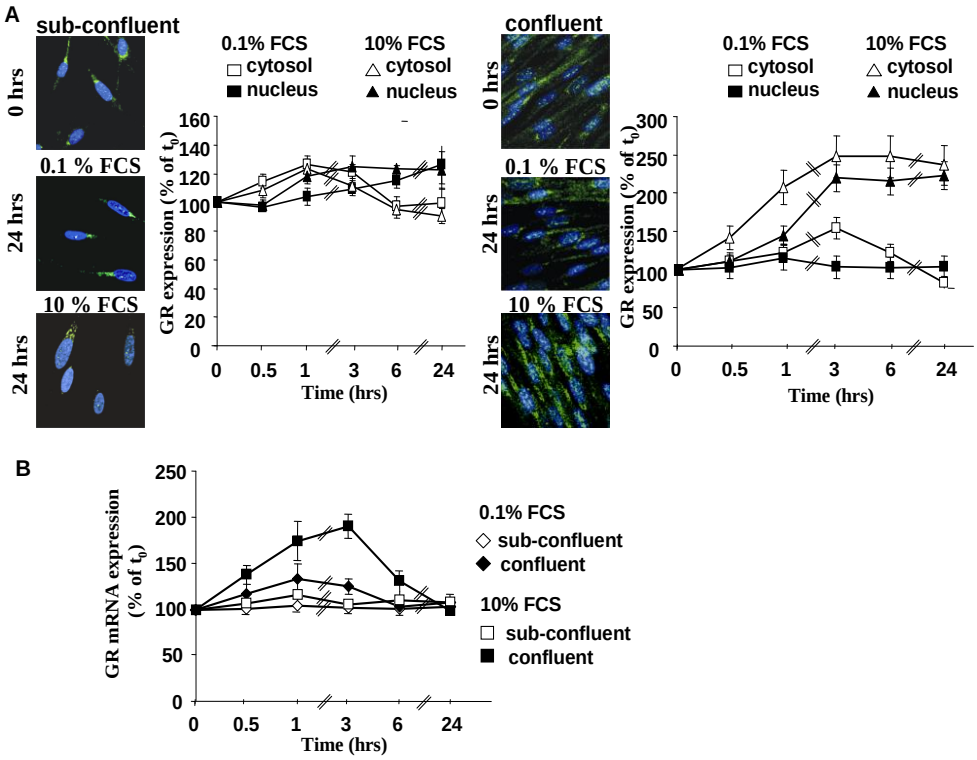

Figure 1. (A) *In the left three representative confocal images we shown the compartment localisation of the GR (green) in the presence and absence of serum (FCS). The nucleus is counterstained with Hoechst dye (blue), and densitometric analysis of immuno-blots of the GR in cytosolic and nuclear protein extracts of sub-confluent fibroblasts. In the three right panels we display representative confocal images of the cell compartment localisation of the GR and densitometric analysis of immuno-blots of the GR in cytosolic and nuclear protein extracts of confluent fibroblasts in the presence and absence of serum (FCS). (B) The effect of serum stimulation on GR mRNA expression. All above presented data points represent the mean ± S.E.M. of triplicate measurements in six different primary fibroblast cell lines.*

Cell density affects serum-dependent C/EBP expression and GR complex formation

Lung fibroblasts expressed two of the four known C/EBP-α isoforms, a 42-45 kDa and a 30 kDa protein (Fig. 3A). In serum deprived cells the majority of both C/EBP-α .isoforms was located in the cytosol and independent of cell density. Interestingly in sub-confluent cells serum induced a fast increase of the 30 kDa C/EBP-α isoform within 3 hours, while the larger isoform was not significantly up-regulated before 18 hours (Fig. 3A, upper panel). In contrast, in confluent fibroblasts serum C/EBP-α was expressed at a much lower level and serum induced a significant increase of the large kDa C/EBP-α isoform at 6 hours declining nearly to basal level within 24 hours (Fig. 3A, lower panel). The 30 kDa isoform was up-regulated at 18 hours and declined also at 24 hours (Fig.3A, lower panel).

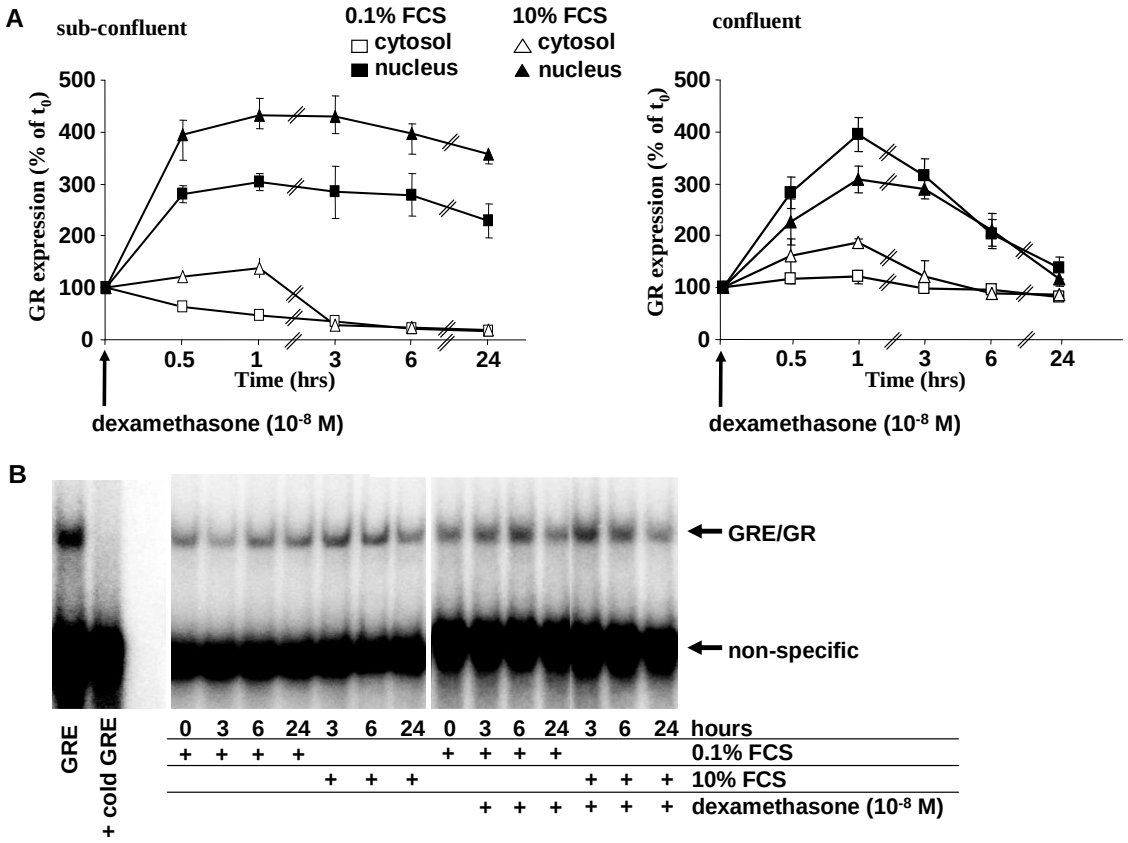

Figure 2. (A) *The effect of dexamethasone on the cell compartmental localisation of the GR in sub-Confluent and confluent fibroblasts and its modification by serum (FCS). Data points represent the mean ± S.E.M. of triplicate measurements in six different primary fibroblast cell lines. (B) A representative EMSA of the GR/GRE complex in confluent fibroblasts and its activation by serum and dexamethasone. Similar results were obtained in three different primary fibroblast lines.*

In serum stimulated sub-confluent fibroblasts dexamethason (10^{-8} M) shifted the large C/EBP-α into the nucleus and the total amount of C/EBP-α was lower at 24 hours compared to start level (Fig.3B). The cell compartmental distribution of the 30 kDa isoform was not shifted by dexamethasone, but its expression was reduced in both compartments (Fig. 3B).

In addition we characterised the expression pattern of C/EBP-β and detected three isoproteins at 45, 40 and 30 kDa with a major band at 45 kDa in the cytoplasma of serum treated sub-confluent and confluent fibroblasts (Fig. 3C). The expression level of all three isoform increased when stimulated with serum and reached peak at 6 hours slowly declining thereafter. The expression of all three C/EBP-β subproteins was higher in confluent cells (n = 4) compared to sub-confluent fibroblasts (n = 4), but neither their expression nor their cell compartmental distribution was affected by dexamethasone (data not shown).

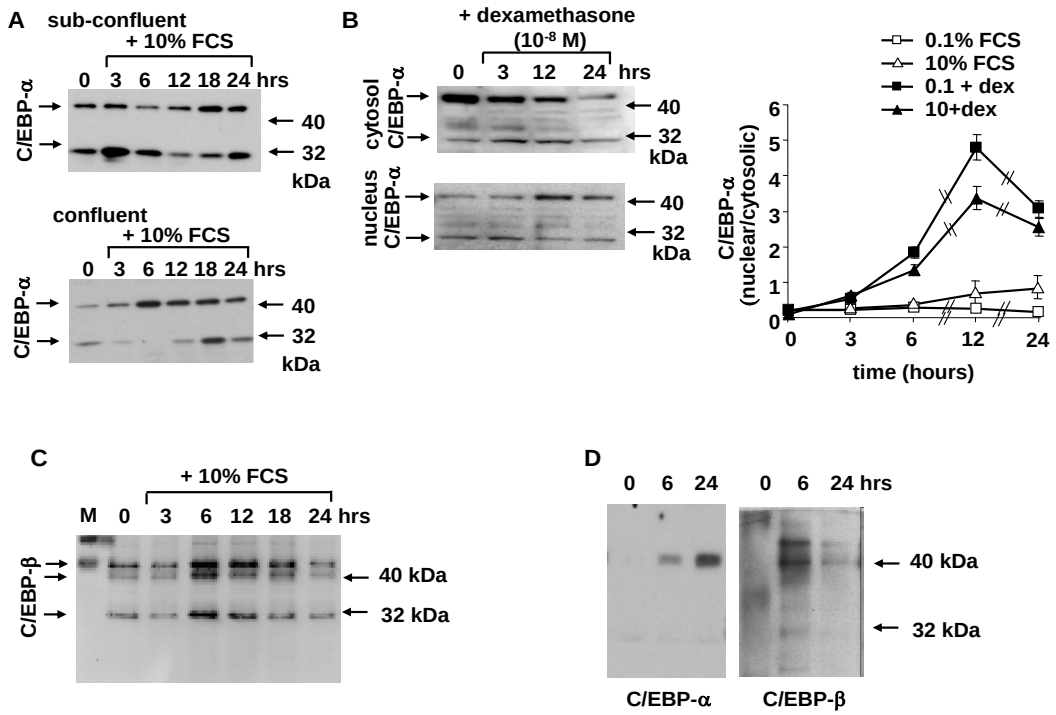

Figure 3. (A) *The effect of cell density of the kinetic of the expression pattern of two C/EBP-α isoproteins (30, 42 kDa) in total cell protein extracts of human primary lung fibroblasts, depicted as a representative immuno-blot. Similar results were obtained in five different fibroblast lines. (B) The dexamethasone induced activation/translocation of the two C/EBP-α isoproteins from the cytosol into the nucleus, depicted as a representative immuno-blot. The ratio of the cytosol / nuclear accumulation of the 42 kDa C/EBP-α were determined by immuno-blots and is shown as mean ± S.E.M. of triplicate measurements in five different primary fibroblast cell lines. (C) Representative immuno-blot of the kinetics of C/EBP-β isoprotein expression in total cell protein extracts of confluent fibroblasts. Similar results were obtained in three other confluent and four sub-confluent fibroblast lines. d Representative immuno-blots of C/EBP-α and –β after the GR was targeted and immnuo-precipitated with an anti-GR antibody from nuclear cell protein extracts 12 hours after the addition of the drug. Similar results were obtained in three other cell lines.*

Using an anti-GR antibody to capture the GR-C/EBP complex followed by immuno-blot analysis of the C/EBP-isoproteins in the precipitates indicated that the complex composition changed over time. At 6 hours a low amount of the 42 kDa C/EBP-α and more of the 40/45 C/EBP-β proteins were co-precipitated with the GR (Fig. 3D). At 24 hours the level of the 42 kDa C/EBP-α was the increased, while that of the two C/EBP-β isoproteins was reduced in the co-precipitates of the GR (Figure 3D).

Cell density and serum modifies the expression of p21$^{(Waf1/Cip1)}$

The kinetics of the GR and C/EBP-α activation overlapped for several hours and should therefore up-regulate the expression of $p21^{(Waf1/Cip1)}$. In sub-confluent serum starved fibroblasts most of the $p21^{(Waf1/Cip1)}$ was located in the nucleus and serum treatment significantly increased its expression within 3 hours ($p<0.05$) declining afterwards (Fig. 4A). In confluent cells serum starved cells we observed no significant changes of $p21^{(Waf1/Cip1)}$ levels, but serum treatment increased its accumulation in the nucleus within 3 hours significantly ($p<0.01$) and maintained it at a high level until 24 hours (Fig. 4A). When fibroblasts were treated with dexamethasone (10^{-8} M) the nuclear accumulation of $p21^{(Waf1/Cip1)}$ decreased to 50% of its initial level ($p<0.01$) 24 hours after the addition of the steroid in sub-confluent cells (Fig. 4B). In confluent cells the steroid caused a significant increase at 6 hours in 0.1% serum ($p<0.01$) and decreased to basal levels at 24 hours (Fig. 4B).

When stimulated with 10% serum and dexamethasone (10^{-8} M) $p21^{(Waf1/Cip1)}$ accumulation increased at 3 hours ($p<0.01$) and maintained at this level until 24 hours in confluent cells (Fig. 4B). The observed increase of $p21^{(Waf1/Cip1)}$ expression by serum or the steroid was preceded by *de novo* synthesis of mRNA (data not shown). Blocking steroid signalling by pre-treating the cells for 30 minutes with either the glucocorticoid receptor antagonist RU486 (10^{-6} – 10^{-8} M) or for one hour with glucocorticoid response element containing decoy-oligo nucleotide sequence (10 μM) inhibited the expression of $p21^{(Waf1/Cip1)}$ (Fig. 4C). We further show that steroid-induced (10^{-6} – 10^{-8} M) inhibition of fibroblast proliferation involved the action of C/EBP-α and $p21^{(Waf1/Cip1)}$, while down-regulation of C/EBP-β or control siRNA for C/EBP-α or $p21^{(Waf1/Cip1)}$ had no significant effect (Fig. 4C).

Cell density and p38 MAP kinase regulate steroid dependent expression of $p27^{(Kip1)}$

Only in confluent fibroblasts steroid treatment (10^{-8} M) induced the expression of a second negative cell cycle regulator, $p27^{(Kip)}$ and this effect was independent of the presence of serum (Fig. 5A). RU486 (10^{-6} M) or GRE decoy oligo-nucleotides (10 μM) diminished the effect of dexamethasone, while siRNA for C/EBP-α or C/EBP-β (1 μM) or decoy for NF-$_{\kappa}$B (1 μM) had no effect (Fig. 5A). Interestingly siRNA for p38 MAP kinase, but not for Erk1/2 MAP kinase (1 μM) significantly reduced the expression of $p27^{(Kip1)}$ in dexamethasone treated

cells (Fig. 5B). Furthermore, in the presence of 10% FCS the expression of $p27^{(Kip1)}$ was up-regulated when the Erk1/2 signaling pathway was blocked (Fig. 5B).

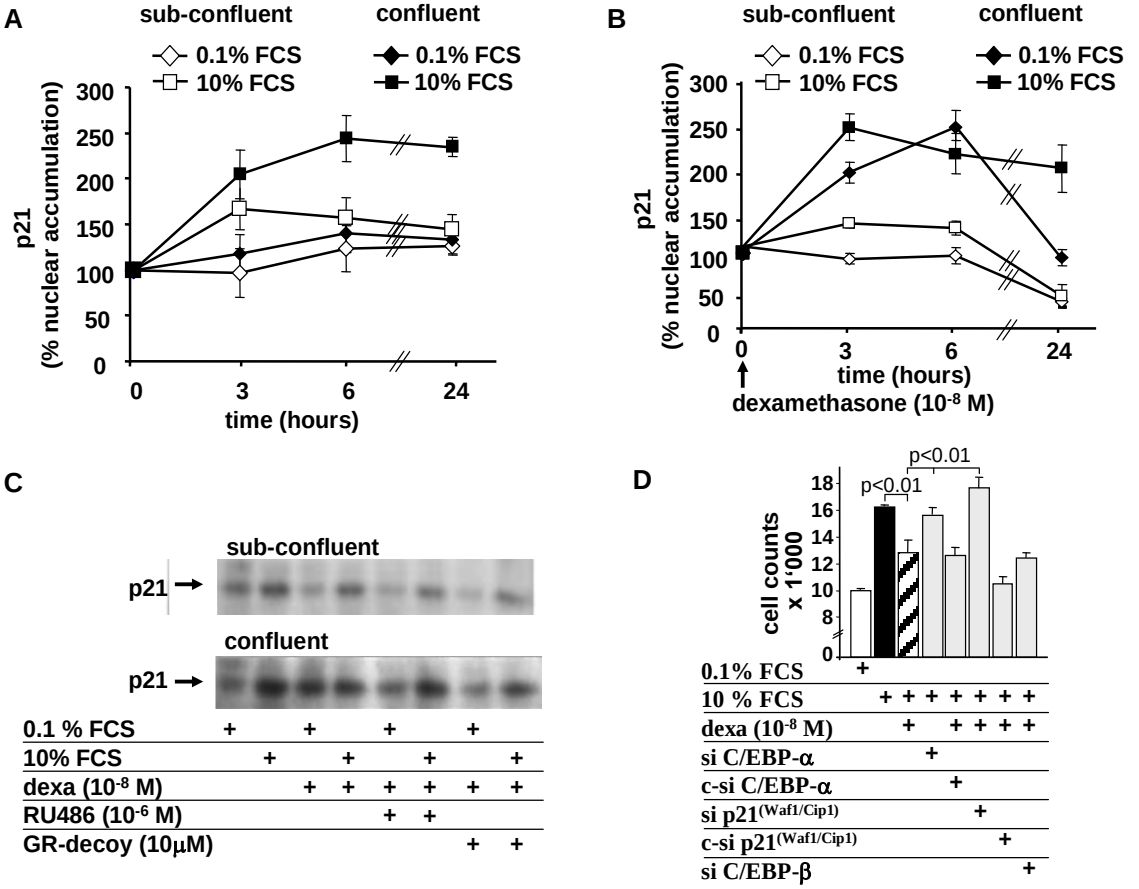

Figure 4. *(A) Kinetic of $p21^{(Waf1/Cip1)}$ expression in nuclear protein extracts of serum deprived and serum stimulated fibroblasts. (B) The effect of dexamethasone on the expression of $^{(Waf1/Cip1)}$ expression and its modification by serum (FCS) in nuclear protein extracts. Data points in panels A and B represent the mean ± S.E.M. of triplicate measurements in five different primary fibroblast cell lines. (C) A representative immuno-blot of the effect of signal pathway inhibitors on the expression of $p21^{(Waf1/Cip1)}$ in nuclear protein extracts. Similar results were obtained in three other cell lines. (D) The effect of dexamethasone, C/EBP-α, - β, and $p21^{(Waf1/Cip1)}$ on serum (FCS) stimulated fibroblast proliferation at the third day. Data points represent the mean ± S.E.M. of triplicate measurements in six different primary fibroblast cell lines.*

We further assessed the role of $p21^{(Waf1/Cip1)}$ and $p27^{(Kip)}$ on proliferation control in confluent contact inhibited cells with and without steroids. As expected cell counts of sub-confluent fibroblast stimulated with 10% FCS increased significantly within 3 days, and a single dose of dexamethasone at day 1 reduced the proliferation dose-dependently (10^{-6}, 10^{-8} M) by maximal 38.2 ± 4.7 % ($p<0.001$). The inhibition of C/EBP-α or $p21^{(Waf1/Cip1)}$ siRNA

diminished the anti-proliferative effect of the steroid, while siRNA for C/EBP-β or $p27^{(Kip1)}$ had no such effect (data not shown). In confluent fibroblasts 10% serum did not significantly increase cell numbers within 3 days and treatment with neither dexamethasone (10^{-6} M, 10^{-8} M), nor $p21^{(Waf1/Cip1)}$, nor C/EBP-α siRNA had any significant effect on cell numbers (Fig. 5C). However, when confluent fibroblasts were treated with siRNA for $p27^{(Kip)}$ the confluent cell numbers further increased significantly ($p<0.02$) over a period of 3 days (Fig. 5C). When $p21^{(Waf1/Cip1)}$ was blocked in addition to $p27^{(Kip)}$ the contact inhibition of cell proliferation was abolished in the presence and absence of the steroid (Fig. 5C).

2.2.5 DISCUSSION

The presented data showed that the expression and the cell compartmental distribution of the GR and its complex formation with the transcription factors C/EBP-α and –β are affected by cell density and the presence of serum. The steroid-dependent expression $p21^{(Waf1/Cip1)}$ requires the presence of 42 kDa C/EBP-α. Only in confluent fibroblasts the steroid induced the expression of $p27^{(Kip)}$ via p38 MAP kinase and a 45/40 kDa C/EBP-β. Contact inhibition of confluent fibroblasts involved the combined action of $p21^{(Waf1/Cip1)}$ and $p27^{(Kip)}$.

Lung fibroblasts play a key role in the pathology of several human diseases including fibrosis, sarcoidosis, chronic obstructive pulmonary disease (COPD), emphysema and asthma (22, 30, 32, 33), and glucocorticoids are the major therapeutic drugs used to control the inflammatory and the pro-fibrotic aspects of these diseases (1). However, neither the contribution of fibroblasts to the pathology of those diseases is clear, nor the benefit of inhaled glucocorticoids on the pro-fibrotic processes has been documented (34). It is widely accepted that the pathological remodelling of the fibrotic lung is similar to badly controlled wound healing. In this study low cell density and serum to mimic he condition of inflammation in which fibroblasts have to fill wounds to reconstitute damaged tissue. We assumed that such a condition represents the initial phase of wound repair when high serum levels are present while being reduced at later stages. Confluent fibroblasts in low serum concentration represented intact undamaged tissue, and the addition of serum mimicked to confluent fibroblasts mimicked the condition found in tissue adjacent to a wound.

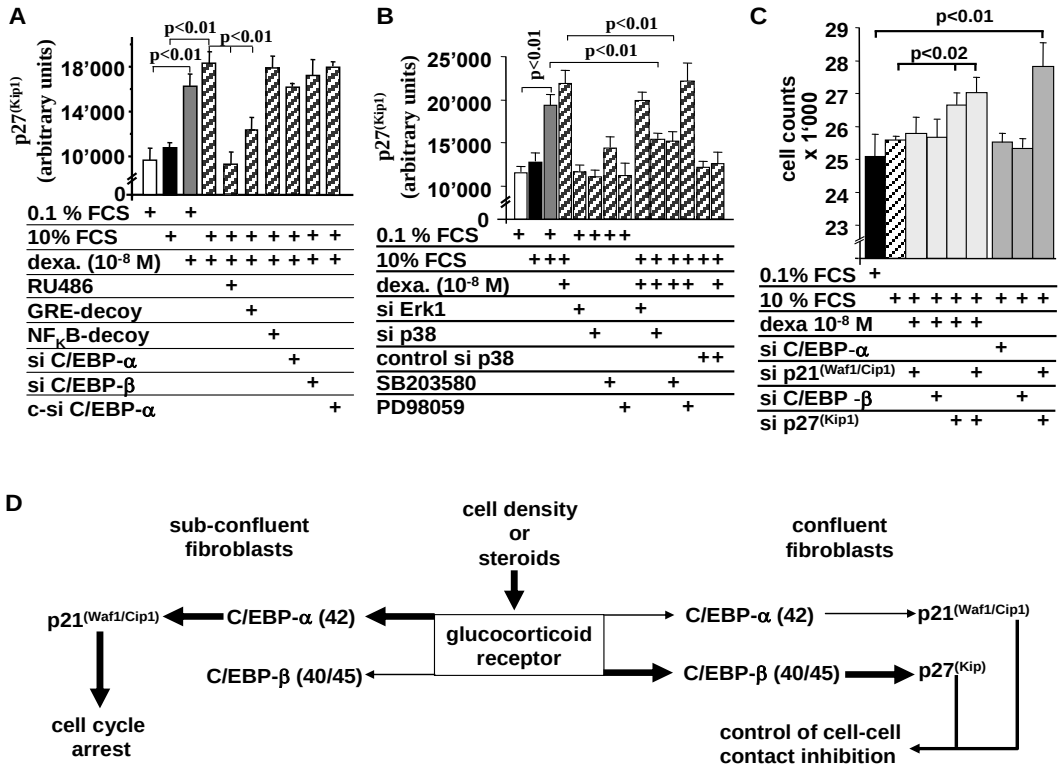

Figure 5. *(A) The role of GR, C/EBP-α and –β signaling on the expression of p27(Kip) in nuclear cell extracts 12 hours after stimulation in confluent fibroblasts. (B) The role of Erk1/2 MAP kinase and p38 MAP kinase signaling on the expression of p27(Kip) in nuclear cell extracts 12 hours after stimulation in confluent fibroblasts. (C) The role of the GR, C/EBP-α, C/EBP –β, p21(Waf1/Cip1) and p27(Kip) on fibroblast proliferation at the third day. Data points in panels A, B, and C represent the mean ± S.E.M. of triplicate measurements in at least four different primary fibroblast cell lines. (D) Summary of the results presented here and interpretation of the function of GR-C/EBP complexes as a consequence of cell density.*

Our data suggest that cell density affects the localisation of the GR in fibroblasts and also modulates its biological availability. The GR is encoded by a single gene which is regulated by three independent promoters producing several GR-isoforms which are further modified by post-translational modifications. The expression of the various GR-isoforms is assumed to be cell type, tissue, or species specific, while their specific function is not well defined (35, 36). The best studied GR-isoforms are GRα and GRβ. It was assumed that the GRβ isoform is only expressed in the nucleus where it competes with the GR-α for GRE binding (36, 37). However, the expression of GRβ is species specific and may be cell type specific (34-37). Based on its characteristic molecular weight we observed only the GRα isoform under all conditions, including confluent fibroblasts and glucocorticoid activation in human fibroblasts.

However, in confluent fibroblasts the nuclear fraction of the GR was significantly increased compared to subconfluent cells. Nevertheless the GRE binding capacity of the nuclear GR isolated from confluent cells was not increased compared to subconfluent cells as assessed by EMSA and ELISA. Dexamethasone increased the GR – GRE binding capacity under all conditions indicating that the nuclear location of the GR in confluent fibroblasts was not caused by increased activation. Cell type and cell layer location specific distribution and activation of the GR is not new and had been described in the placenta (38). Interestingly, the authors observed that extended treatment with glucocorticoids resulted in a 90% down-regulation of GR expression in placenta fibroblasts, which is comparable with the effect we observed in lung fibroblast cultures. In our experiments the consequence of GR depletion was the loss of the anti-proliferative effect of the glucocorticoid.

In this study we provide evidence that the complexes that are formed by the GR and members of the C/EBP family is affected by cell density and that the composition of the complex seems to regulate the effect of steroids on the cell cycle regulator that is subsequently activated. While a GR-C/EBP-α complex results in $p21^{(Waf1/Cip1)}$ expression, that of the GR with C/EBP-β seems to lead to $p27^{(Kip)}$ activation. We further provide evidence that the 30kDa isoproteins of both C/EBPs are not significantly binding to the GR. The anti-proliferative effect of glucocorticoids is clearly linked with the activation of C/EBP-α and $p21^{(Waf1/Cip1)}$ (10, 15-18), while the necessity of p53 for $p21^{(Waf1/Cip1)}$ gene expression is not clear (10, 38), and could also be a feedback mechanism of p53 on GR expression (31, 39). Interestingly, the nuclear localisation of accumulation of $p21^{(Waf1/Cip1)}$ extended the life-span of senescent human fibroblasts in the presence of dexamethasone (40) and in long term treated cells of Cushing syndrome patients (18). Together with the observation that in pancreatic fibroblasts nuclear $p21^{(Waf1/Cip1)}$ was associated with a phenotypic switch of the cells into myo-fibroblasts (29) suggests that $p21^{(Waf1/Cip1)}$ in confluent fibroblasts may have different properties compared to proliferating sub-confluent cells or may need additional factors to control proliferation.

In addition to $p21^{(Waf1/Cip1)}$ dexamethasone induced the expression of a second cell cycle control protein $p27^{(Kip)}$, which has been reported in other cell types before (41) and may depend on residue specific phosphorylation or dimerisation of the GR (42). Again the interaction of $p21^{(Waf1/Cip1)}$ with $p27^{(Kip)}$ may be cell type or species specific (3, 15, 37, 43). In our experiments deprivation of $p21^{(Waf1/Cip1)}$ expression alone in confluent serum exposed fibroblasts was insufficient to overcome contact inhibition. Only when $p27^{(Kip)}$ was also

down-regulated confluent serum stimulated fibroblasts lost contact inhibition and grew in multiple layers, the effect could not be stopped by dexamethasone treatment. The interaction of $p27^{(Kip)}$ with $p21^{(Waf1/Cip1)}$ in regard to cell proliferation control had been described by others (15, 37) and may even make cell cycle arrest independent of cyclin dependent kinase 2 (3, 43).

In figure 5D we provide a schemata which summarises our findings, which imply that in sub-confluent fibroblasts the GR forms a complex with the 42 kDa C/EBP-α which leads to $p21^{(Waf1/Cip1)}$ expression and cell cycle arrest, while in confluent cells the GR binds preferably to the 45/40 kDa C/EBP-β isoproteins which induces $p27^{(Kip)}$ expression. This data further supports the idea that the function of the GR is modified by cell differentiation and inflammation and needs to be further addressed in the context of pathologies that either develops steroid resistance or that do not respond well to steroid therapy.

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CHAPTER 3

Tachyphylaxis and the prolonged inhibitory effect of mometasone on lung tissue remodelling

Glucocorticoids are very effective in asthma treatment, but in long-term usage they cause severe side effects and can result in reduced expression and function of the GR assigned as tachyphylaxis. This in turn leads to reduce steroid response.

Mometasone has recently been introduced to asthma treatment. Compared to other steroids it is chracterised by a long lasting action at low doses and less frequent application. Like other steroids, it penetrates the cell membrane passively, binds to the GR, followed by translocation into the nucleus where it acts as a transcription factor. However as other steroids the mometasone activated GR can also interact with other transcription factors, such as AP-1 and NF-$_K$B to expand its functions. The mechanisms of the action of mometasone are not fully understood yet.

Lung mesenchymal cell activation and proliferation importantly contribute to the pathogenesis of inflammatory lung diseases, here we used 5% fetal calf serum (FCS) in fibroblasts and smooth muscle cells to mimic inflammation.

3.1 OBJECTIVES:

In this study I compared the effect of the classical glucocorticoid, dexametasone to that of the long acting glucocorticoid mometasone on:

1. the effect of mometasone on the expression and activation of the GR,
2. the effect of mometasone on the expression and activation of C/EBP-α,
3. the effect of mometasone on the expression and activation of $p21^{(Waf1/Cip1)}$,

4. the effect of mometasone on the expression and activation of matrix metalloproteinases,

5. the effect of mometasone on extracellular matrix deposition,

6. the anti-proliferative capacity of mometasone on serum activated primary human lung fibroblasts and smooth muscle cells.

3.2 Tachyphylaxis and the prolonged inhibitory effect of mometasone on lung tissue remodelling

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At a Glance Commentary: In this study we found that mometasone was a more potent inhibitor of cell proliferation and keeps the glucocorticoids receptor for a prolonged period activated. Potentially these differences are caused by reduced or absent tachyphylaxis of the glucocorticoid receptor after treatment with mometasone. Our findings might be relevant for therapy of inflammatory lung diseases with high dose or repeated steroids.

3.2.1 Abstract

Rationale: The long term steroid therapy can be impaired by reduced expression and function of the glucocorticoid receptor, tachyphylaxis. **Objectives:** In this study we compared the effect of dexamethasone to long acting mometasone on the expression and activation of the glucocorticoid receptor, C/EBP-α, $p21^{(Waf1/Cip1)}$, matrix metalloproteinase, extracellular matrix deposition, and their anti-proliferative capacity in primary human lung fibroblasts and smooth muscle cells. **Measurements and Main Results:** Protein expression was determined by immuno-blotting and gelatine zymography, proliferation by thymidine incorporation. Dexamethasone induced glucocorticoid receptor activity decreased significantly faster at 6 hours compared to that induced by mometasone at 24 hours. Similarly drug specific effects were observed for the expression of C/EBP-α and $p21^{(Waf1/Cip1)}$. The anti-proliferative effect of mometasone was significantly stronger, long lasting, and could not be washed out compared to dexamethasone. Furthermore, when treated with mometasone a second dose on day two further reduced fibroblast proliferation, while dexamethasone had no such effect. Similar anti-proliferative effects were observed in bronchial and vascular smooth muscle cells. Regarding airway remodelling we observed that mometasone, in contrast to other steroids, did further stimulate serum dependent synthesis and deposition of extracellular matrix and did not affected matrix metalloproteinase-2 and -9 expressions or activity. **Conclusions:** Our findings indicate that mometasone does not induce tachyphylaxis and has a preferable effect on airway remodelling. Therefore mometasone might help to reduce steroid dosage in asthma therapy and thereby reduce the risk of tachyphylaxis and tissue remodelling. (Words: 237)

3.2.2 Introduction

Airway remodelling is a pathology shared by most chronic inflammatory lung diseases (1-4). Tissue repair processes of the airway wall's sub-mucosa include increased extracellular matrix deposition and increased numbers of (myo-) fibroblasts and smooth muscle cells altering lung function (2, 4).

Novel long lasting steroids such as mometasone have been introduced to asthma therapy to reduce steroid dosage and application frequency (5). Mometasone has a prolonged bioavailability in the lung (6, 7), and its long term systemic effect on cortisol reduction is lower than other steroids (8, 9). All steroids penetrate the cell membrane passively, bind to the glucocorticoid receptor (GR), which then migrates into the nucleus to act as a transcription factor (10, 11). Steroids, including mometasone, inhibit other transcription factors, AP-1 and NF-$_K$B (12) which is substrates for proliferative signal transducers (13). This action might also inhibit the AP-1 dependent histone acetylation that is necessary for gene transcription (14), and may explain partly mometasone's anti-proliferative effect. Furthermore, mometasone activates the CREB binding protein, a histone acetylase, and controls DNA methylation in NF-$_K$B activated genes (15).

The deregulation of fibroblast and bronchial smooth muscle cell function in the airway sub-mucosa may be a cause for lasting structural changes in chronic inflammatory lung diseases which increase long-term morbidity (2, 16 - 18). However, it is unclear whether infiltrating activated immune cells stimulate the fibrotic process, or if their infiltration follows the release of cytokines by lung resident cells. Therefore lung mesenchymal cell activation and proliferation importantly contributes to the pathogenesis of inflammatory lung diseases, but is often neglected as a therapeutic aim.

Steroids in general have a potent anti-proliferative effect (10, 19), but for mometasone the information is limited (20). Airway wall stiffness and flexibility correlates with the accumulation of extracellular matrix (ECM), and may be increased by classical steroids in inflamed tissue and a beneficial effect of steroids on airway wall remodelling would not occur in the inflamed lung (21). Furthermore, in fibroblasts steroids down-regulate GR expression within hours which might result in a decreased response to the drugs in long-term, tachypylaxis (22-24). Steroid response can also be lost by proteasomal GR protein degradation (25) or by suppression of *de novo* synthesis at the transcriptional level (26), such

effects are most probably independent of the steroid's glucocorticoid receptor-binding affinity and may be drug specific.

The aim of this study was to compare the anti proliferative effect and the underlying molecular mechanism a short acting steroids to that a long-acting steroid in primary human lung mesenchymal cells of non-asthmatic adults. We also compared the effect of classical steroids to mometasone on ECM deposition and used 5% serum to mimic inflammation (21).

3.2.3 Materials and Methods

Cell culture and experimental conditions: Human lung fibroblast, pulmonary vascular and bronchial smooth muscle cells were isolated and grown as described earlier (27-29). Experiments were performed between 3^{rd} to 7^{th} cell passages. Prior to treatment cells ($10^4/cm^2$) were serum deprived (0.1% FCS) for 24 or 36 hrs. RU486 or steroids were added 30 min prior to other treatments.

Cell proliferation: Serum deprived cells were stimulated (5% FCS) and [^{3}H]-thymidine incorporation was determined during the last 8 hrs and quantified by liquid scintillation counting (19).

Immuno-histochemistry: Cells grown on cover slips were washed 3x with phosphate buffered saline (PBS) and fixated with methanol: acidic acid (3:1). Slides were washed twice before endogenous peroxidase was inhibited (0.1% NaN_3, 0.3% H_2O_2), washed twice and unspecific antibody binding was blocked (5% FCS, 0.05% Tween-20 in PBS; 1 hr). Slides were incubated overnight with 1:400 diluted GR antibodies (sc-1003, Santa Cruz Biotechnology, CA) in blocking buffer (4 ℃), washed twice and incubated with horseradish peroxidase-labelled antibodies (sc-2004, 1:1000, blocking buffer, 1 hr). Slides were washed 3x with PBS and stained using an AEC Chromogen Kit (AEC101, Sigma, Saint Louis, MO). Images were documented by Analysis-D soft imaging system (Olympus IX50 microscope, objective 40x). The same procedure was performed with 1:300 diluted $p21^{(Waf1/Cip1)}$ antibodies (610234, BD Biosciences Pharmingen, San Diego, CA) and 1:1000 diluted horseradish peroxidase-labelled antibodies (sc-2005).

Nuclear and cytosolic extracts and immuno-blotting: Cytosolic and nuclear proteins were prepared and the protein-concentration was determined by Bradford's method as described earlier (19). Total protein (10 μg) was size-fractionated in a denaturing 4-15% SDS-PAGE and transferred onto PVDF membranes (Millipore Corp., MA); controlled by Ponceau's staining (19). Membranes were incubated in blocking buffer (10mM Tris, 150mM NaCl, 0.05% Tween-20, 5% skimmed milk) for 1 hr, followed by overnight incubation (4 ℃) with either glucocorticoid receptor, C/EBP-α, or $p21^{(Waf1/Cip1)}$ specific antibodies (sc-8992, sc-61, sc-6246; Santa Cruz Biotech). Unbound antibodies were washed off and membranes were incubated (30 min) with horseradish peroxidase-labelled species specific antibodies (Chemicon). Protein bands were detected by ECL (Pierce, IL) for evaluation of optical density (19).

Extracellular matrix synthesis and gelatinase activity: Deposition of extracellular matrix was determined by [^{3}H]-proline incorporation (21) and gelatinases MMP-2 and -9 were detected by gelatine zymography (30).

Reverse transcriptase–polymerase chain reaction (RT-PCR): Total RNA was extracted using RNeasy® Mini kit (Qiagen, Basel, Switzerland) and 1 μg was transcribed into cDNA using murine leukemia virus reverse transcriptase (Promega, Madison, WI). PCR primers used were: GR forward 5'-CACCCTCACTGGCTGTCGCTTCTC-3', reverse 5'-TGA CAAACGAAAGAGGAGACCGCC-3', 23 cycles: denaturation: (98°C, 20 sec), annealing (58 ℃, 22 sec), extension (72 ℃, 30 sec); p21 (primer set, R&D Systems Inc, MI); β2-microglobulin (β2-M): forward 5'-CTCGCGCTACTCTCTCTCTTTCT-3', reverse 5'-TTAAGTGGGATCGAGACATGTAAGC-3', 23 cycles: denaturation (98°C, 30 sec), annealing (60 ℃, 60 sec), extension (72 ℃, 60 sec). PCR products were analysed by electrophoresis (1% agarose gel) stained with ethidium bromide (21).

Statistics: Statistics were performed using SPSS 11.5.1. (SPSS Inc., Chicago, IL). Student's t-test was used for densitometric data and ANOVA for proliferation, with $p<0.05$ indicting significance from H_0-hypothesis of equal distribution.

Additional details for all methods are provided in data supplement

3.2.4 Results

Dexamethasone, budesonide, and fluticasone had a similar dose-dependent anti-proliferative effect and inhibited serum-induced (5% FCS, 24 hrs) [^{3}H]-thymidine incorporation significantly ($p<0.05$) at concentrations > 10^{-7} M with no significant difference comparing the drugs to each other (figure 1A). Compared to dexamethasone mometasone significantly decreased fibroblast proliferation at a lower a concentration > 10^{-10} M ($p<0.05$; figure 1A). The anti-proliferative effects of all four steroids was blocked by 30 min pre-incubation of fibroblasts with 10^{-6} M RU486 (figure 1A). Assessing the anti-proliferative effect of the four steroids in two other lung cell types we observed similar drug specific effects in bronchial smooth muscle cells (figure 1B) and pulmonary vascular smooth muscle cells (figure 1C). As in fibroblasts the occupation of the GR by RU486 inhibited the anti-proliferative effect of all steroids (figure 1B, 1C). Since we observed no significant anti-proliferative differences comparing budesonide or fluticasone to dexamethasone we performed all subsequent experiments comparing dexamethasone to mometasone in lung fibroblasts.

Kinetic of GR trafficking and glucocorticoid specific down-regulation. GR activity was determined by immuno-blotting and immuno-histochemistry. In serum-starved cells and in cells treated with 5% FCS the GR was located in a restricted cytosolic closed to the nuclear membrane (figure 2A). One hour after addition of a steroid (10^{-8} M) a significant amount of the GR was shifted into the nucleus. Based on densitometric analysis we observed no significant differences comparing the effect of dexamethasone (557±16 optical units: OU) to mometasone (503±34 OU), however compared to 5% FCS (397±36 OU) the effect of both steroids was significant with a $p<0.01$ (figure 2B). Furthermore, six hours after the addition of dexamethasone the expression of the GR in the nucleus (288±19 OU) was significantly reduced ($p<0.01$) compared to mometasone (475±30 OU). When the incubation of fibroblasts with steroids was extended to 24 hrs dexamethasone treated cells showed a further down-regulation of GR expression (254±23 OU), while this effect in the presence of mometasone (425±18 OU) was significantly lower ($p<0.01$) (figure 2B). We also could confirm that dexamethasone treatment was followed by proteosomal degradation of the GR as the steroid dependent down-regulation of GR expression was inhibited when the cells were pre-treated with the 26 S proteasome inhibitor MG-132 (figure 2A).

Immuno-blotting of nuclear protein extracts confirmed the drug specific kinetic of GR activation and down-regulation (figure 2C). A summary of the densitometric analysis of the

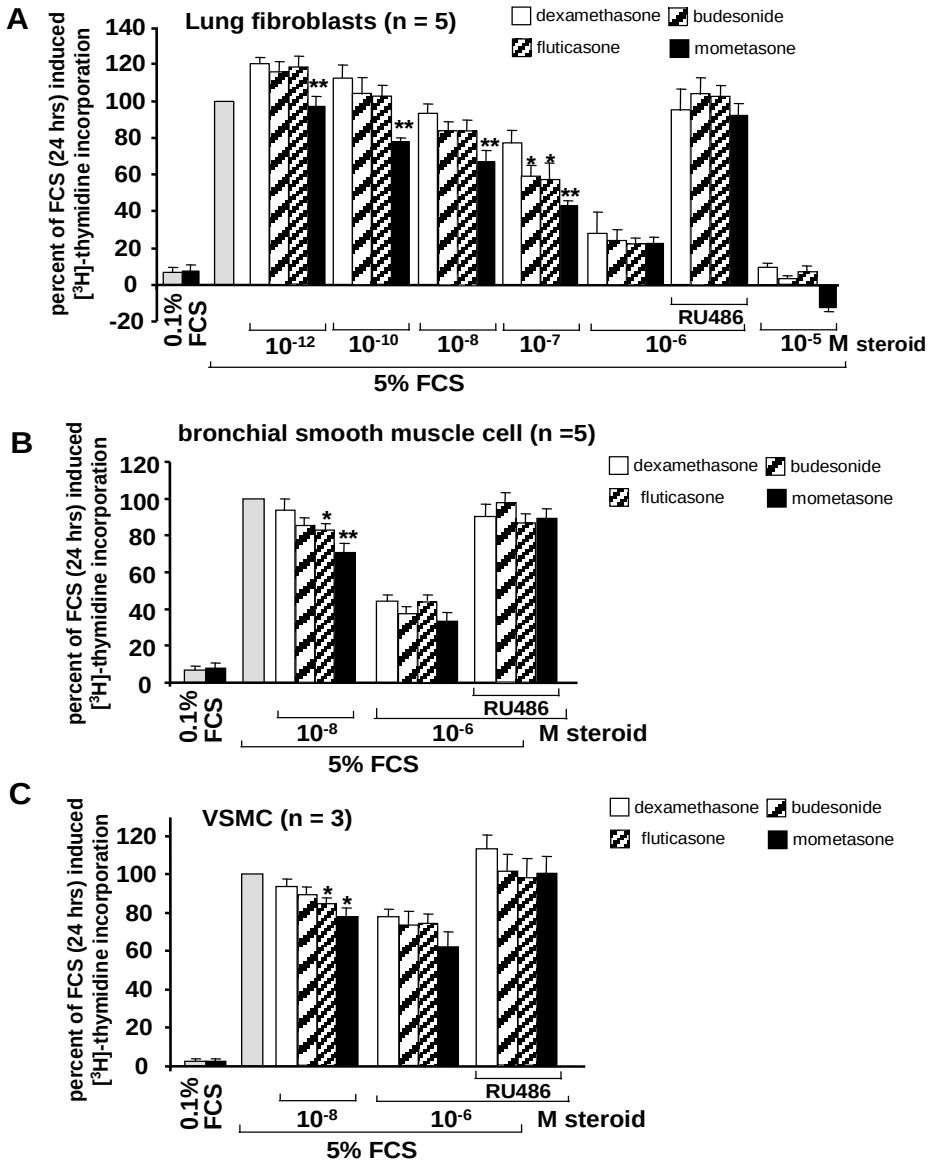

Figure 1*: (A) Dose-dependent inhibition of serum-stimulated lung fibroblast proliferation by different steroids. To determine proliferation fibroblasts ($10^6/cm^2$) were serum starved for 24 hours (0.1% FCS) before proliferation was stimulated by 5% FCS. The drugs were added together with the growth medium (5% FCS containing RPMI 1640) and were present over the entire test period. To determine the involvement of the GR in the anti-proliferative effect of steroid cells were pre-incubated for 30 minutes with 10^{-6} M RU486, prior to the addition of one of the steroids. (B) Similar experiments were performed in primary human bronchial smooth muscle cells and (C) in pulmonary vascular smooth muscle cells. Bars represent the mean ± S.E.M. calculated for the indicate number of cell lines. Experiments were performed at least in duplicate in each cell line. For all graphs statistically significant differences (paired, two-tailed student's t-test) of steroid compared to 5% FCS stimulated cells are indicated by: *: p< 0.05; **: p < 0.01.*

accumulation of GR in the nucleus in five different human lung fibroblast cell lines is shown in figure 2D. Both steroids caused a fast increase in nuclear GR accumulation within 1 and 3 hrs and a subsequent significant decline of the GR signal at six hours in the presence of dexamethasone (57±17% vs. control, $p<0.05$), but not with mometasone (132±16% vs. control).

In addition we analysed the effect of the two steroids on the expression of total GR. When fibroblasts were cultivated in the presence of a steroid for 16 hrs the overall expression of the GR was significantly lower compared to control cells in the presence of 5% FCS and, this decrease is more pronounced in the presence of dexamethasone (10^{-8} M) compared to mometasone (10^{-8} M) (figure 2E). We did not observe a significant effect of any treatment on the ratio of the GR-α to GR-β (figure 2E). Furthermore, we could confirm that the proteasome inhibitor MG-132 significantly inhibited the steroid dependent degradation of the GR (figure 2E).

In addition we determined the effect of the two steroids on the mRNA transcription of the GR. The expression level of the mRNA encoding for various genes of interest was determined by RT-PCR as a ratio to β-actin mRNA expression. Cells that were kept in starving medium (0.1% FCS) were used as control for each time point. Stimulation of fibroblasts with 5% FCS-induced an increase expression of the GR mRNA significantly at all time points after 3 hrs, and both steroids significantly down regulated this effect until 24 hrs. However, in the presence of mometasone GR mRNA levels dropped less compared to dexamethasone and at 6 hrs started to incline again reaching approximately 50% of the 5% FCS induced level (figure 3A). The inhibitory effect of the two steroids on the GR mRNA was not observed when the cells had been pre-treated with RU486 (10^{-6} M) for 30 min (data not shown).

To further elucidate the signaling pathway that mediates the anti-proliferative action of the two steroids we compared the effect of the two steroids on the transcription of C/EBP-α and $p21^{(Waf1/Cip1)}$ in human lung fibroblasts and airway smooth muscle cells. In figure 3B we show that 5% FCS induced an increase of the transcription of C/EBP-α within 3 hrs and declined after 3 hrs; this effect was not significantly altered by the steroids, even so mometasone seemed to extend the active period for 6 hrs. In the same samples we examined

the expression of the mRNA encoding for p21$^{(Waf1/Cip1)}$ which was not stimulated by 5% FCS, but in the presence of any of the two steroids at 6 and 24 hrs. Mometasone induced a stronger signal of p21$^{(Waf1/Cip1)}$ at all time points compared to dexamethasone (figure 3C).

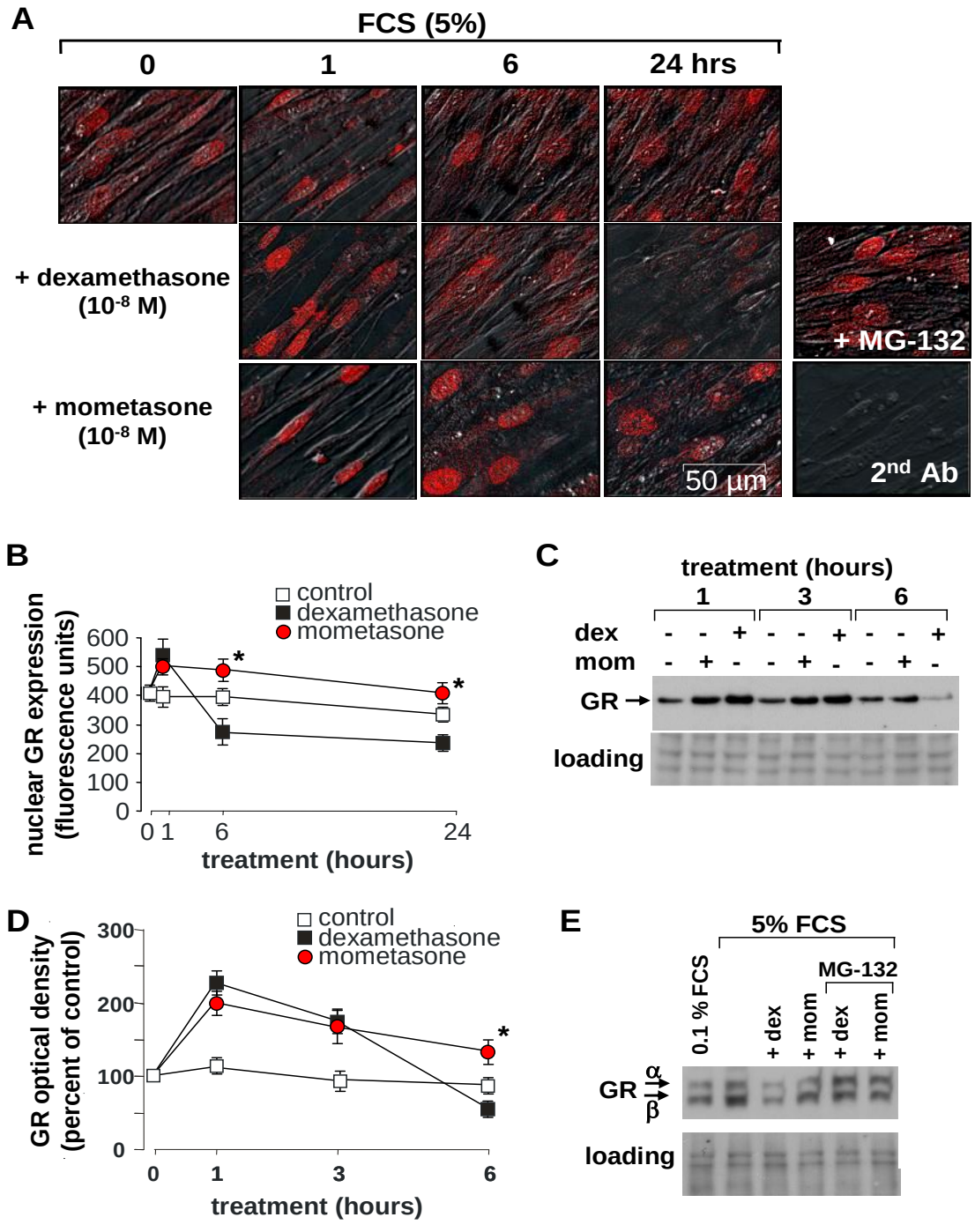

Figure 2. (A) Representative photographs of the cell compartmental distribution of the GR in 5% FCS stimulated fibroblasts (upper panel row), or in cells treated with 10^{-8} M dexamethasone (middle row) or 10^{-8} M mometasone (lower row). The effect of the proteasome inhibitor MG-132 (10^{-6} M) on dexamethasone stimulated GR depletion is at 24 hours (middle row, last picture). Unspecific staining due to the 2nd antibody is presented at the end of the mometasone treatment. (B) Time course of the nuclear accumulation of the GR determined by densitometrical analysis of confocal microscopy (100 cells were analysed for each set) in 5% FCS treated

*control fibroblasts versus treatment with dexamethasone (10^{-9}M) or mometasone (10^{-8} M). Each data point represents the mean ± S.E.M. calculated for 5 independent fibroblast cell lines. * indicates a statistic significant difference with $p < 0.05$ comparing the effect of dexamethasone (10^{-8} M) or mometasone (10^{-8} M) to 5% FCS by paired, two tailed student's t-test. (C) Representative Immuno-blot of GR accumulation in the nuclear protein fraction of fibroblasts and its stimulation by dexamethasone (dex) or mometasone (mom) at a concentration of 10^{-8} M. Similar results were obtained in four additional fibroblast lines. (D) Semi-quantitative analysis of the kinetic of steroid induced nuclear GR accumulation relative to un-treated cells. Each data point represents the mean ± S.E.M. of five fibroblast lines. * indicates a statistic significant difference with $p < 0.05$ comparing the effect of dexamethasone (10^{-8} M) or mometasone (10^{-8} M) to 5% FCS by paired, two tailed student's t-test. (E) The effect of dexamethasone (dex) and mometasone (mom) on the expression of total GR and isoforms in human lung fibroblasts. Similar results were obtained in four other lines.*

The function of C/EBP-α and $p21^{(Waf1/Cip1)}$ depends on their cell compartmentalisation, therefore we assessed the localisation of both proteins before cells were treated with any steroid and 24 hrs later. In figure 3D we show representative immuno-histochemical staining for both factors under different culture conditions. In resting cells we observed no C/EBP-α, while 24 hrs in 5% FCS increased the signal in the cytosol, and it was shifted into the nucleus when treated with one of the steroids (10^{-8} M) (figure 3D).

Compared to 0.1% FCS treatment 5% FCS for 24 and 48 hrs did not induce any increase of $p21^{(Waf1/Cip1)}$ protein expression, which was expressed at a similar level in the cytosol as well as in the nucleus (figure 3D). Stimulation with dexamethasone or mometasone (10^{-8} M) induced a significant increase of total $p21^{(Waf1/Cip1)}$ and also in the nucleus within 24 hrs (figure 3D). Nuclear accumulation of $p21^{(Waf1/Cip1)}$ was maintained for 48 hrs when fibroblasts were treated with mometasone, while cells treated with dexamethasone expressed $p21^{(Waf1/Cip1)}$ levels that were similar to 5% FCS alone (figure 3D).

To determine the duration of the anti-proliferative effect after a single dose of a steroid, fibroblasts were treated with one of the steroids (10^{-7} M) for 24 hrs. The cells were than washed twice with PBS and normal growth medium was added for an additional 24 hrs. As depicted in figure 4A fibroblasts that had been treated with mometasone proliferated significantly slower compared to cells treated with dexamethasone. RU486 (10^{-6} M) counteracted the anti-proliferative effect of mometasone and dexamethasone (figure 4A). Since dexamethasone and mometasone had different effects on the GR expression and mRNA synthesis at 24 hrs we investigated if the anti-proliferative effect of either steroid was affected by pre-treatment with steroid. Therefore cells were either treated for 24 hrs with 10^{-8} M dexamethasone or mometasone. On the second day the culture medium was replaced and new steroids added for additional 24 hrs. The treatment protocol day1 – day2 was either: a single

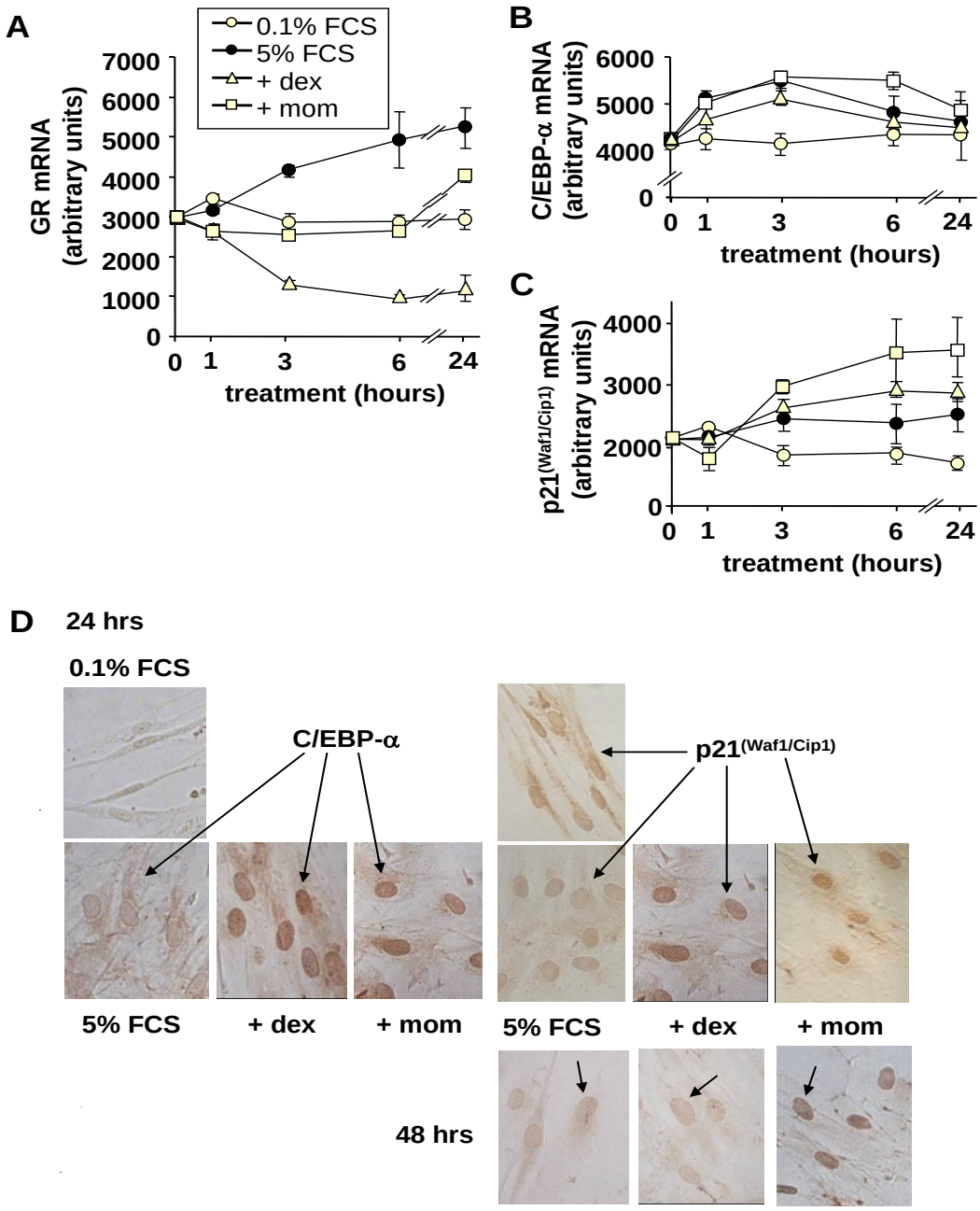

Figure 3: (A) *The effect of serum starving (0.1% FCS), serum stimulation (5% FCS) and steroid treatment (dex = 10^{-8} M dexamethasone; mom = 10^{-8} M mometasone) on the expression of GR mRNA determined by RT-PCR over a period of 24 hours. Each data point represents the mean ±S.E.M. of five fibroblast lines. (B) The expression of C/EBP-α encoding mRNAs was determined in the same cDNA preparations used in panel A. (C) The expression of p21$^{(Waf1/Cip1)}$ encoding mRNAs was determined in the same cDNA preparations used in panel A. (D) Representative immuno-staining for the effect of 10^{-8} M dexamethasone (dex) or 10^{-8} M mometasone (mom) on the expression and cell compartmental localisation of C/EBP-α (24 hours) and p21$^{(Waf1/Cip1)}$ (24, 48 hours) in sub-confluent serum stimulated (5% FCS) fibroblasts. Similar results were obtained in three additional cell lines.*

dose of dexamethasone or mometasone for 48 hrs, or dexamethasone (24 hrs) followed by a medium change with fresh dexamethasone (24 hrs), and similarly for the combinations: mometasone / mometasone, or dexamethasone / mometasone, or mometasone / dexamethasone. Thymidine incorporation was determined at day1 and day2 during the last 8 hrs of cell culture. As shown in figure 4B dexamethasone treatment had a short lasting anti-proliferative effect,

while mometasone under all circumstances was stronger anti-proliferative drug. The results also indicate that mometasone did not deplete the GR as it had an additive effect when given twice or was followed by dexamethasone; furthermore, RU486 inhibited this effect (data not shown).

As reported earlier (21) in details dexamethasone reduced extracellular matrix deposition in the absence of serum and increased the stimulating effect of serum (figure 4C). Mometasone also reduced the deposition of extracellular matrix in the absence of serum, but did not further increase its synthesis and deposition in the presence of serum (figure 4C). The inhibitory effect of both steroids was mediated by the GR as it could be inhibited when cells were pre-treated with RU486 30 min prior to steroid addition (figure 4C). We further investigated the effect of the two steroids on extracellular matrix metabolism assessing the expression and activity of major gelatinases MMP-2 and MMP-9 in the cell culture medium of steroid treated and un-treated cells at 12, 24, 36, and 48 hrs after addition of the drugs. None of the two steroids affected the expression or activity of MMP-2 or MMP-9 within 48 hrs (figure 4D).

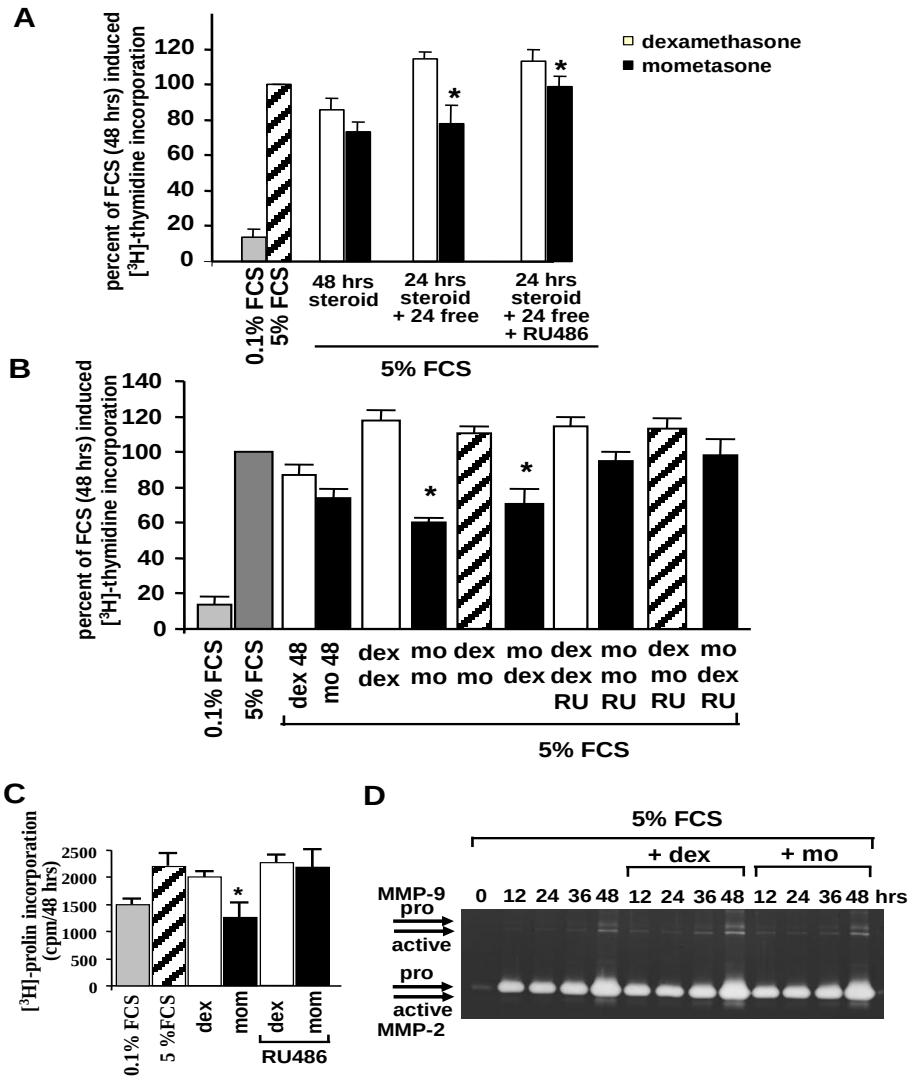

*Figure 4: (A) The effect of repeated steroid treatment on serum induced (5% FCS) fibroblast proliferation over 48 hours. (B) The effect of the alternated treatment of dexamethasone (dex) with mometasone (mom) and the role of GR depletion by a short acting steroid (dexamethasone) on proliferation. (C) The involvement of the GR in dexamethasone (dex) and mometasone (mom), both 10^{-8} M, on serum (5% FCS) induced deposition of extracellular matrix by lung fibroblasts (n = 3) at 48 hours. For all experiments presented in graphs A-C, bars represent the mean ± S.E.M. calculated for at least independent fibroblast lines, and each experiment was performed in triplicate; and RU486 was used at a concentration of 10^{-6} M and cells were 30 minutes pre-incubated before the addition of the first steroid. * indicates a statistic significant difference with $p < 0.05$ comparing the effect of dexamethasone (10^{-8} M) to that of mometasone (10^{-8} M) by paired, two tailed student's t-test. (D) A representative gelatine zymography of the effect of 10^{-8} M dexamethasone (dex) or mometasone (mom) on the expression and activation of the gelatinases MMP-2 and MMP-9 by human lung fibroblasts. Similar results were obtained in two additional fibroblast lines.*

3.2.5 DISCUSSION

In this study we demonstrate that mometasone, compared to dexamethasone, has a stronger and prolonged anti-proliferative effect *in vitro* on primary human lung fibroblasts, vascular and bronchial airway smooth muscle cells. In fibroblasts the prolonged effect correlates with a extended activation of the GR and a long lasting induction of C/EBP-α and the anti-proliferative protein $p21^{(Waf1/Cip1)}$ by mometasone at therapeutic achievable concentrations. The decrease of GR expression after steroid treatment was due to the inhibition of *de novo* mRNA synthesis and to proteosomal degradation of the GR protein. However, the degradation of the nuclear GR was significantly delayed when stimulated with mometasone compared to dexamethasone and may therefore reduce the occurrence of tachyphylaxis. Furthermore, we provided evidence that different from other steroids mometasone does not increase extracellular matrix accumulation in the presence of serum (inflammation).

In agreement with others our data showed that the GR was expressed by serum starved growth arrested fibroblasts and accumulated on the cytosolic side of the nuclear membrane. In absence of steroids the GR travels without DNA-binding throughout the cell, but does not bind to the glucocorticoid response element (GRE) consensus sequence (31). In our experiments mometasone induced a fast and prolonged activation of the GR compared to dexamethasone. This observation is to a certain extend opposing an earlier study which reported no significant differences of the activation of the GR compared to another steroid, but suggested that mometasone activates the mineralocorticoid and progesterone receptors in addition (32). However, there are other additional mechanisms by which mometasone could achieve its specific profile of effects as it inhibits the action of other transcription factor including AP-1 and NF-κB (12-15). We have reported earlier that the anti-proliferative effect of steroids in lung fibroblasts involves the action of the transcription factor C/EBP-α which subsequently activates the expression of the cell cycle control protein $p21^{(Waf1/Cip1)}$ (33). Our data shows that mometasone induced a similar kinetic as dexamethasone for the activation of C/EBP-α, however, it may induce a longer lasting availability as the synthesis of C/EBP-α mRNA was prolonged by three hours. Thus the extended activation period of the GR together with the availability of active C/EBP-α would extend the time frame in which both factors could form the complex which is essential to induce $p21^{(Waf1/Cip1)}$ expression. Such an effect

would be similar to the mechanism that we described earlier for the interaction of long acting β_2-agonists with steroids (19). Indeed the nuclear accumulation and therefore the activation of $p21^{(Waf1/Cip1)}$ was extended by mometasone treatment compared to dexamethasone, suggesting a longer anti-proliferative effect of mometasone.

Steroid induced down-regulation of the GR, tachyphylaxis, has previously been described for cortisol, prednisone, dexamethasone, fluticasone and budesonide in various cell types (34-37). The degradation of the GR after stimulation with a steroid, including mometasone is due to proteosomal protein degradation as it was significantly reduced by the proteasome inhibitor MG132 or when ubiquitination was inhibited (25,38). However, the kinetic of GR down-regulation may be cell type specific or depend on cell culture conditions. In COS-1 and Hela cells dexamethasone-induced degradation of the GR involved ubiquitination and disintegration by proteasome 26 (25). Similarly, in this study, dexamethasone treatment of human lung fibroblasts significantly decreased the abundance of the GR that was overcome by the proteasome inhibitor MG132. This effect did not affect total GR expression, but specifically the GR accumulation in the nucleus (25). In contrast to dexamethasone, mometasone did not down-regulate the nuclear accumulation of the GR over 24 hrs. This difference of mometasone can not be explained by its 12 x stronger binding affinity to the GR as in fact the nuclear signal of the GR appeared to be stronger at early time points in the presence of dexamethasone. The prolonged activation of the GR by mometasone could be an important feature mediating its prolonged anti-proliferative effect.

In human lung tissue, including human lung fibroblasts GR tachyphylaxis has not been studied, yet. In this study we demonstrated that normal non-transformed human lung fibroblasts are susceptible to GR tachyphylaxis, but the strength of this effect depends on the steroid used. Drug specific differences in nuclear retention of agonist-activated GR have been described comparing GR-GRE complexes induced by various synthetic steroids, but not for a long acting synthetic steroid such as mometasone (31, 39, 40). Trypsin digestion of such steroid-ligated GRE complexes showed distinct degradation products indicating drug-induced alterations of the complex with the consequence of different enzyme sensitivity and therefore distinct stability (41). Our data indicate that the ligand's property may change the structure of the GR-GRE complexe, thereby modifying its sensitivity to degradation in the nucleus. Either this effect or a stronger steroid-GR binding could confer a protection of the protein-DNA complex from endoplasmatic reticular and cytosolic proteasome degradation (42). In this context, we showed that the anti-proliferative effect of mometasone is resistant to depletion of

the steroid by wash out experiments which again indicated a difference in the complex formed by the mometasone with the GR and the GRE. Furthermore, the prolonged and stronger anti-proliferative effect of mometasone was also observed in pulmonary vascular smooth muscle cells and bronchial smooth muscle cells indicating a general rather than a cell type specific effect.

We also observed a significant difference for the re-synthesis of the GR after activation by dexamethasone compared to mometasone. When treated with dexamethasone the signal for the GR mRNA declined after 6 hrs and stayed at low level over 24 hrs. In contrast, when fibroblasts were treated with mometasone the mRNA for the GR increased after an initial decline and was above serum stimulated levels at 24 hrs, indicating that mometasone did not reduce GR re-synthesis, and therefore thachyphylaxis may be reduced or counterbalanced.

The anti-proliferative effect of short acting steroids involves the activation of C/EBP-α, which forms a complex with the active GR which seems to be essential for the subsequent expression of the cell cycle inhibitor $p21^{(Waf1/Cip1)}$ (33, 43). This mechanism seems also to be apply for the anti-proliferative action of mometasone, but with a drug specific extended expression and activation phase of both C/EBP-α and $p21^{(Waf1/Cip1)}$, which may explain the prolonged anti-proliferative action of mometasone.

Lung remodelling in asthma and COPD not only depends on the proliferation of (myo-) fibroblasts and airway smooth muscle cells; it also includes the deposition of extracellular matrix by the same cell types (2-4, 18). Surprisingly, only few studies investigated the effect of steroids and other anti-asthmatic drugs on this aspect. Unfortunately their results were contradictory and can hardly be compared as the drugs used in each study were different, and the period of treatment and dosage were not identical (44-49). However, we have shown in fibroblasts that several steroids have a dual effect of extracellular matrix deposition and turn over (21). When confluent cell layers of human lung fibroblasts were treated with a steroid alone in the absence of serum the deposition of extracellular matrix and collagens was significantly reduced, while when the steroid was given in the presence of 5% serum it further increased the synthesis and deposition of the extracellular matrix and collagens (21). Here we demonstrate that mometasone has a different effect on extracellular matrix deposition, as in contrast to dexamethasone it did not increase serum stimulated extracellular matrix deposition. However, whether this effect will reflect in a beneficial reduction of airway wall thickening has to be proven in clinical studies. In addition we provide data that implies that

the effect of steroids, including mometasone, on the turn over of the extracellular matrix does not involve the action of gelatinases MMP-2 or MMP-9.

In conclusion we found that mometasone was a more potent inhibitor of cell proliferation and keeps the GR for a prolonged period activated. Potentially these differences are caused by reduced or absent tachyphylaxis of the glucocorticoid receptor after treatment with mometasone. Our findings might be relevant for therapy of inflammatory lung diseases with high dose or repeated steroids.

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Chapter 4

The long acting effect of ciclesonide on mesenchymal cells depends on its activation by epithelial cells

Ciclosonide is a very effective long acting glucocorticoid which has been introduced to therapy of asthma. Clinical studies have shown that a single inhalation of ciclesonide per day is sufficient to control asthma. Ciclesonide as such carries a butyl-ester group and cannot bind to the GR directly, only after partial digestion by esterases its active metabolite desisobutyryl-ciclesonide is formed. Only this active metabolite of ciclesonide can bind to the GR and has been shown to activate the same signaling pathway as the classical steroids. In the bronchi, ciclesonide gets in contact to esterase synthesized by airway cells and is thus activated at the lung. It is un-known if any other cell type beside epithelial cells of the lung are capable to activate ciuclesonide. Furthermore, the esterfication of ciclosonide is a revertible process, thus the butyl-group can be exchanged by longer fatty acids creating a more lipophilic cellular stock of the drug within the cell. Such an effect could modify the bio-availability of ciclesonide and therefore its time of action.

4.1 OBJECTIVES:

1. Does ciclesonide induce a long activation of the GR compared to other long acting and short acting glucocorticoids?

2. Does the active metabolite of ciclesonide induce a long acting activation of the GR?

3. Which cell types of the human lung can metabolize/activate ciclesonide into its active form?

4.2 The long acting effect of ciclesonide on mesenchymal cells depends on its activation by epithelial cells

4.2.1 Summary:

Long term therapy with inhaled glucocorticoids in asthma often results in reduced drug response, due to reduced expression of the glucocorticoid receptor. Long acting glucocorticoids were developed to overcome such side effects. In asthma therapy glucocorticoids reduce the secretion of pro-inflammatory mediators and airway remodelling. Here we investigated the effect of ciclesonide and its active metabolite on glucocorticoid receptor activation and on cell proliferation, of bronchial smooth muscle cells. The cicelsonide metabolite-1, budesonide or fluticasone activated the glucocorticoid receptor fast and with a short duration of the active phase, Non-metabolised ciclesonide had neither a significant effect on glucocorticoid receptor activity nor did it inhibit cell proliferation. The kinetic of the glucocorticoid receptor activation by the ciclesonide metabolite was similar to that of classical glucocorticoids. Co-culture experiments of bronchial smooth muscle cells together with epithelial cells revealed that ciclosonide was converted into its active form by epithelial cells and that it than was inhibiting the proliferation of bronchial smooth muscle cells. Furthermore, in the co-culture system the glucocorticoid receptor was activated for extended periods in bronchial smooth muscle cells. All inhibitory actions of the ciclesonide metabolite or ciclesonide in the co-culture system could be blocked by pre-incubation of the cells with RU486 indicating that the drug's mode of action is mediated via the glucocorticoid receptor. In summary our data indicate that the molecular mechanism underlying the long lasting effect of ciclesonide depends on its metabolisation by epithelial cells and the action of its active metabolite is not different from that of classical glucocorticoids.

4.2.2 Introduction

Glucocorticoids act via binding to their intracellular glucocorticoid receptor (GR) which is activated, dimerizes, and translocates into the nucleus where it acts as transcription factor. The GR also achieves a part of its effects through its interaction with other transcription factors, such as C/EBP-α, C/EBP-β, Stat3/5, NF-κB, or AP-1. Most of these transcription factors have been shown to be involved in the control of cell proliferation (Rogatsky and Ivashkiv, 2006c;Bruna et al., 2003;Zhang et al., 1997;Floyd and Stephens, 2003;Aljada et al., 1999;Gotoh et al., 1997;Adcock et al., 1996a;Borger et al., 2002;Cha et al., 1998).

Glucorcoiticoids are most potent and consistently effective in reducing airway hyperresponsiveness, improving lung function and decreasing symptom severity (Georgitis, 1999). Inhaled glucocorticoids such as fluticasone and budesonide are frequently used to control symptoms in moderate to severe asthma (Edmonds et al., 2002). The continuous use of high dose glucorcoticoids, however, can result in severe side effects—impaired growth in children, decreased bone mineral density, skin thinning and bruising, and cataracts. (Rosen and Miner, 2005;Dahl, 2006). Various glucocorticoids are known to activate the GR and their potency has been established looking at side effects or binding capacity to the GR (Umland et al., 2002;Newton, 2000). Little is known about the duration of GR activation by various glucocorticoids after *in vivo* and *in vitro* application (Umland et al., 2002).

The potential advantage of ciclesonide over "classical" steroids is its long action. Clinical studies showed that for treatment with ciclesonide a single inhalation per day is sufficient to control asthma (Kanniess et al., 2001;Pearlman et al., 2005). Different from glucocorticoids such as budesonide or fluticasone, the drug ciclesonide carries a butyl-ester group which keeps it in a conformation that does not function as an active glucocorticoid. Ciclesonide is activated after in halation locally by enzymatic partial digestion by esterases which form its active metabolite, desisobutyryl-ciclesonide (des-CIC) (Furthwängler et al., 2002;Silvestri et al., 2006;Reynolds and Scott, 2004). In the bronchi, ciclesonide gets in contact to esterase synthesized by airway cells and is thus activated at the lung area which is mainly affected by the inflammation during an asthma attack. Interestingly, esterfication of a glucocorticoid is a revertible process, thus the butyl-group can be exchanged by longer fatty acids creating a more lipophilic cellular stock of the drug within the cell and such a process would re-create the inactive form of ciclesonide intracellular, which might further contribute to its extended

bio-availability (Edsbäcker and Brattsand, 2002;Reynolds and Scott, 2004). It is assumed as a beneficial effect of ciclesonide that the portion of the inhaled drugs that precipitates in the pharynx wall, and thus is swallowed, is considered to be inactivated as it is digested in the liver (Furthwängler et al., 2002). Finally, des-CIC is converted into a series of inactive metabolites that are eliminated from the body (Hansel et al., 2006;Peet et al., 2005;Ukena et al., 2006b;Silvestri et al., 2006).

The only other synthetic glucocorticoid that is known to be a metabolite of esterases is budesonide (Edsbäcker and Brattsand, 2002). However, in contrast to ciclesonide, budesonide is applied as the active, non-esterized form (Edsbäcker and Brattsand, 2002). However, in the treatment of persistent asthma ciclesonide has been shown to be more effective compared to budesonide (Ukena et al., 2006a). Epithelial cells are suggested to be the main source of esterases and synthesis and activation of esterases by other human lung cells such as fibroblasts or smooth muscle cells is speculative (Edsbäcker and Brattsand, 2002;Furthwängler et al., 2002;Christie, 2004;Silvestri et al., 2006) .

Therefore in this study we assessed the duration of the GR by ciclesonide and its active metabolite in human epithelail cells, fibroblasts and bronchial smooth muscle cells. We determined the anti-proliferative efficacy of both compounds and their effect on extracellualar matrix deposition as an indicator for tissue remodeling. In addition we investigated the role of epithelial cells on the activation of ciclesonide in a co-culture system.

4.2.3 Methods

All chemicals were from Calbiochem (Ca, USA) unless otherwise stated. RU486 was from Russel-Uclaf, France, Budesonide from AstraZeneca (Sweden), fluticasone from GlaxoSmithKline (UK), ciclesonide and ciclesonide metabolite I from Altana, Germany.

Cell culture: Primary human lung fibroblasts, bronchial smooth muscle cell (BSMC) and epithelial cell cultures were established from human lungs obtained from patients undergoing lobectomy for therapeutically reason as described earlier in details (Johnson et al., 2001;Tamm et al., 2001). All drugs were solubilized in DMSO (Merck, Darmstadt) and further diluted using RPMI/0.3 % albumine as potential carrier for lipophilic glucocorticoids. Drug solutions were not further sterile filtered.

Bronchial smooth muscle cell culture: Primary cultures of human BSMC were grown from airway muscle bundles obtained from 4 probands. Cells were grown in RPMI (Seromed) containing 10 % fetal calf serum (FCS, Invitrogen), and 20 mM HEPES (Seromed). All experiments were performed between passages 3 – 6. Prior to experiments, cells were serum starved for 48 hours using RPMI-1604 containing 0.1 % FCS. No antibiotics were used at any time.

Epithelial cell culture: Primary cultures of human bronchial epithelial cells (BEC) were established form brushings of the epithelium cell layer from 4 patients. Cells were grown in epithelial cell medium (Cambrex, Netherlands) supplemented with insulin, T4, cortisol, bovine pituitary gland extract, and insulin according to the instructions of the manufacturer (all Cambrex). Primary cell cultures were performed on lysine coated cell culture dishes (BD BEC cultures were propagated by trypsination, and cells were transferred into collagen I / fibronectin (80 µg/cm^2) coated cell culture dishes (BD) or cover glass plates (VWR international AG, Switzerland). Prior to experiments cells were starved for 48 hours in cortisol free epithelial medium. No antibiotics were used at any time.

Nuclear and cytosolic extracts: Cytosolic extracts were prepared from 80 % confluent cells resuspended in low salt buffer [20 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM $NaVO_4$, 1 mM EDTA, 1 mM EGTA, 0.2 % NP-40, 10 % glycerol, Complete™ protease inhibitor (Roche Diagnostics, Switzerland)] and incubated for 10 min on ice. After centrifugation for 1 min. at 13‘000 x g (4^{o}C) supernatants were taken as cytosolic fraction. The remaining pellet was dissolved in 50 µl high salt buffer (420 mM NaCl, 20 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM $NaVO_4$, 1 mM EDTA, 1 mM EGTA, 20 % glycerol, Complete^{TM} protease inhibitor) and samples were kept on ice for 30 min, followed by a centrifugation step at 13‘000 x g for 10 min (4 ^{o}C). Supernatants were taken as nuclear fraction (Eickelberg et al., 1999). Protein-concentration was measured according to Bradford's method (Biorad).

Western Blot: To determine expression of GR protein 5-15 µg total protein were size-fractionated in a denaturing 4-15 % SDS- polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto PVDF membrane (Millipore Corp., MA, USA) by a semi-dry transfer. Membranes were blocked for 1 hour (10 mM Tris, 150 mM NaCl, 0.05 % Tween 20, 5 % skimmed milk) and incubated with polyclonal antibodies specific for GR (E20, Santa Cruz) at 4 ℃ over night, followed by incubation with 2nd HRP-linked anti-rabbit antibody (cat# AP160B, Chemicon) for 30 min. Protein bands were detected by enhanced chemiluminescence (ECL, Pierce, IL, USA). GR expression in all samples was done by scanning of X-ray films. Optical density (OD) of GR-signals was evaluated by using AIDA 2.0 and ImageJ Ver 1.32c. Western blots were normalized according to Eschenhagen et al. (Eschenhagen et al., 1992).

Electrophoretic mobility shift assay (EMSA): DNA binding proteins were characterized by EMSA using a [^{32}P]-labelled GRE oligonucleotide (ABR). Binding conditions: NaCl 20 mM, KCl 60 mM, $MgCl_2$ 5 mM, DTT 20 mM, dIdC 1 µg/µg nuclear extract, 1 µg BSA/µg nuclear extract, and incubation was carried out for 30 min. at 21 ℃. DNA/protein complexes were analysed on a non-denaturing 6 % polyacrylamide gel, and exposed to a phosphor imager screen. Specificity of the observed DNA/protein complexes was characterised by pre-incubating the extracts with a 50 fold excess of unlabeled GRE.

Immunohistochemistry: Cells were grown on cover slips (VWR). After treatment, cover slips were rinsed with ice-cold phosphate buffered saline (PBS; 5 min.) and fixed with 4% formaldehyde at room temperature (20 min.). If not otherwise stated, all chemicals were from Sigma (Buchs, Switzerland). After washing twice with PBS, slides were placed in a humid chamber and unspecific binding was blocked (coat of PBS, 5 % goat serum, 0.3% Triton-X-100) and incubated for 1 hour at room temperature. Then, slides were incubated at 4 ℃ with polyclonal rabbit anti-GR antibodies (sc-1003, Santa Cruz Biotechnology) diluted 1:200 in 1 % goat serum in blocking buffer overnight. After two further washes with PBS, slides were incubated with 150 µl of Cy3 labelled goat anti-rabbit antibody (Jackson Immuno Research Laboratories, Inc; 1:300 dilution in 1 % goat serum blocking buffer) and incubated at room temperature (1 hour), in a dark and humid chamber. Stained slides were then washed twice with PBS and twice (5 minutes) with distilled water before being embedded in Fluorsave^{TM} (Calbiochem). Images were captured using confocal imaging at 543 nm, LP 585 nm (Zeiss, Jena, Germany). For all pictures analysed, same acquisition settings were used. To avoid influence of photo bleaching, areas were searched using light microscopy and pictures were

then directly taken using confocal laser microscopy. Stacks were opened in ImageJ Ver. 1.32c and nuclear staining was measured in the stack with the strongest signal.

[^{3}H]-Thymidine incorporation: PDGF or FCS-induced cell proliferation was determined by [^{3}H]-thymidine incorporation. [^{3}H]-thymidine incorporation was determined in 60-80 % confluent cells that had been serum-deprived previously for 36 hrs (0.3 % albumine, exchanged every 12 hrs) followed by PDGF (10 ng/ml) or FCS (5%) stimulation for 24 hrs in the presence or absence of either glucocorticoid. Ru486 was from Roussel Uclaf (Romainville, France), MG 132 was from Calbiochem. After treatment, drugs were not removed until cells were harvested. Five µCi/ml [^{3}H]-thymidine (Amersham, Little Chalfont, UK) were added to culture medium for the last 8 hrs. Cells were harvested on glass fiber paper (GF/C-Unifilter, Canberra Packard Instruments, Meriden, CT, USA) using an automatic cell harvester (Filtermate 186, Canberra Packard Instrument). Incorporated radioactivity was measured with a liquid scintillation counter (Topcount, Canberra Packard Instruments) and values were displayed and analysed as counts per minute (cpm).

For Co-culture experiments, mesenchymal cells were cultured in 6 well transwell plates with a layer of A549 cells in the Transwell. Steroid was added to the transwell. Before harvesting, A549 cells were removed. Cells were washed 3 times with PBS followed by cell lysis using 0.3 M NaOH and liquid scintillation counting.

4.2.4 Results:

Ciclesonide is a weak activator of glucocorticoid receptor activation

Compared to other steroids such as budesonide and fluticasone, ciclosonide is a weak stimulator of the glucocorticoid receptor (figure 1). Budesonide and fluticasone induced a complete depletion of the glucocorticoid receptor signal from the cytosol and shifted the glucocorticoid receptor into the nucleus within 30 minutes. Fluticasone, induced the fastest and strongest accumulation of the glucocorticoid receptor in the nuclear protein fraction, but the signal also weakened fastest in the presence of flucticasone (figure 1).

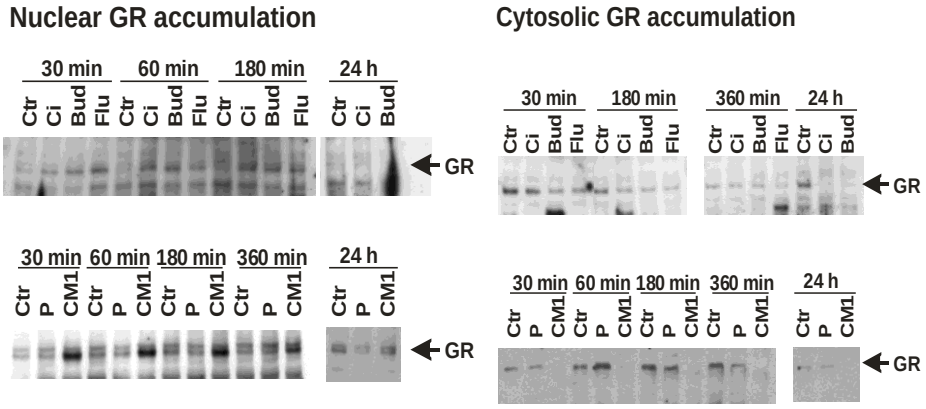


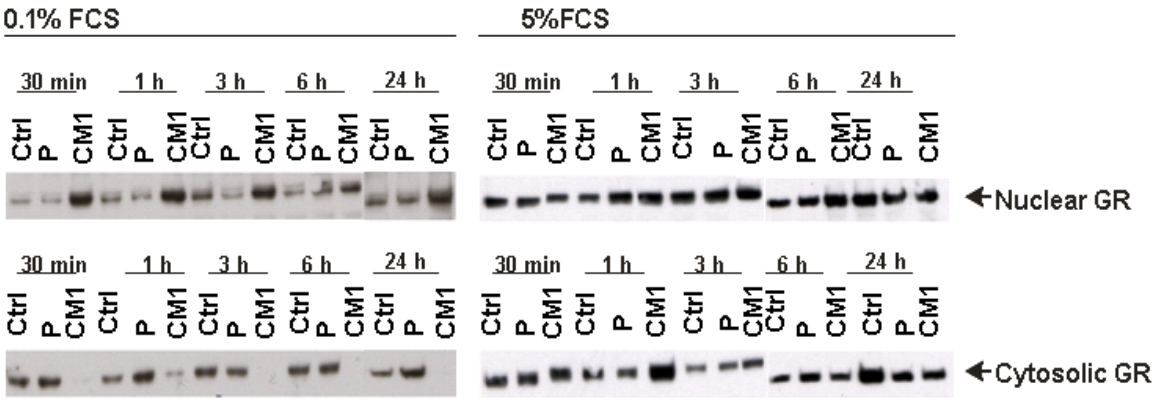
Figure 1 *First line describes the time point of cell harvesting. Lanes indicate: Ctr – starving medium, Ci – ciclesonide, Bud – budesonide, Flu – fluticasone, P – PDGF, CM1 – ciclesonide metabolite I. All drugs were used at 10-8 M. The arrow depicts the specific band of the GR.*

In contrast, only a slight increase of the glucocorticoid receptor in the nuclear protein fraction was seen in the presence of ciclesonide after 1 hour.

As shown in figure 1B. The cilcesonide metabolite-1 was a strong activator of the glucocorticoid receptor. The shift of the glucocorticoid receptor from the cytosol into the nucleus was complete after 30 minutes and was stronger than the effect of busdesonide or fluticasone at the same concentration. The ciclesonide metabolite-1 induced nuclear accumulation of the glucocorticoid receptor followed a similar kinetic as that of budesonide

(figure 1). However, after 24 hours the signals of the glucocorticoid receptor signal in all steroid treated cells was completely reduced and reach back ground level in both cellular compartments (figure 1).

We have described earlier that cell density and specifically the presence of a general pro-inflammatory factor such as serum modifies the bio-availability of the glucocorticoid receptor. Therefore we tested the effect of 5% FCS on the activation of the glucocorticoid receptor by



***Figures 2**, Incubation of ASMC with ciclosonide metabolite 1 induced a shift of GR from the cytosol to the nuclear compartment. ASMC cultures with 0.1% and 10%FCS.First line describes the time point of cell harvesting. Lanes indicate: Ctr – starving medium, P – PDGF, CM1 – ciclesonide metabolite I. All drugs were used at 10(-8) M. The arrow depicts the specific band of the nuclear GR and cytosolic GR.*

steroids. As shown in figure 2, serum induced a rapid increase of the glucocorticoid receptor within 30 minutes in both the cytosol and the nucleus. Due to the strong effect of serum alone we could not determine any additional increase of the glucocorticoid receptor signal in the presence of steroids at early time points. Interestingly the cytosolic glucocorticoid receptor signal increased in the presence of the ciclesonide metabolite-1 at 30 minutes and 1 hour after drugs addition (figure 2). At three and six hours the signal of the glucocorticoid receptor in the nucleus was significantly stronger when the cells were stimulated with the ciclesonide metabolite-1 compared to control cells and of cells that had been stimulated with PDGF-BB, however this effect disappeared at 24 hours (figure 2). The total amount of glucocorticoid receptor however, did not diminish after steroid treatment in the presence of serum, a finding that is in line with our earlier study on the effect of serum on glucocorticoid receptor expression in lung fibroblasts.

The activation of the glucocorticoid receptor was also determined by immuno histochemistry and confirmed the drug specific effect on glucocorticoid receptor activation. We already described above that ciclesonide has to be converted into its active metabolite by the action of esterases. Epithelial cells are considered as the major source of estareses in the lung. Therefore we first determined the activation of the glucocorticoid receptor by ciclesonide in epithelial cells using immuno-histochemistry, and compared the activation of the glucocorticoid receptor to budesonide and fluticasone The Epithelial cells were starved in steroid free medium for 12 hours before addition of the respective steroids at 10^{-8} M. In untreated cells, the GR is mainly located in the cytosol. Fluticasone or budesonide let to rapid translocation of the GR into the nucleus of the cells within 10 to 30 minutes. In contrast, ciclesonide treatment did not lead to GR translocation before six hours. After 24 hours treatment, it is no different on the GR translocation with budesonide, fluticasone and ciclesonide all at 10^{-8}M in epithelial cells. Treatment of epithelial cells with ciclesonide leads to a slow activation of the GR when compared to that stimulated by either budesonide or fluticasone. This finding suggests that primary human bronchial epithelial cells are effectively activating by a slow metabolism of ciclesonide into its active metabolite (figure 3).

The functional activation of the glucocorticoid receptor by ciclesonide metabolite-1

Next we assessed whether the nuclear accumulation of the glucocorticoid receptor was paralleled by its increased binding affinity to its consensus DNA sequence the glucocorticoid response element, GER. Using electrophoretic mobility shift assay (EMSA) with the GRE sequence as a binding target for the glucocorticoid receptor we were able to demonstrated that all steroids induced a fast activation and DNA binding activity of the glucocorticoid receptor. Budesonide and fluticasone led to activation of the GR as fast as 30 minutes after treatment. GR activated by fluticasone reached its peak as early as 30 to 60 minutes and declined at 6 hours. Ciclesonide metabolite-1 was able to mediate a persistent GR activation over 24 hours. Budesonide-mediated GR activation lasted for up to 6 hours. But at 24 hours GR activity was about 20 % higher in ciclesonide metabolite-1 treated cells compared to budesonide. In the cytosolic fraction, glucocorticoid treatment led to a slight increase in the presence of activated GR of airway smooth muscle cells. This was measurable over 24 hours. However, since activated GR is rapidly transferred into the nucleus, detection of activated GR within the cytosol is close to the detection level of EMSA (figure 4).

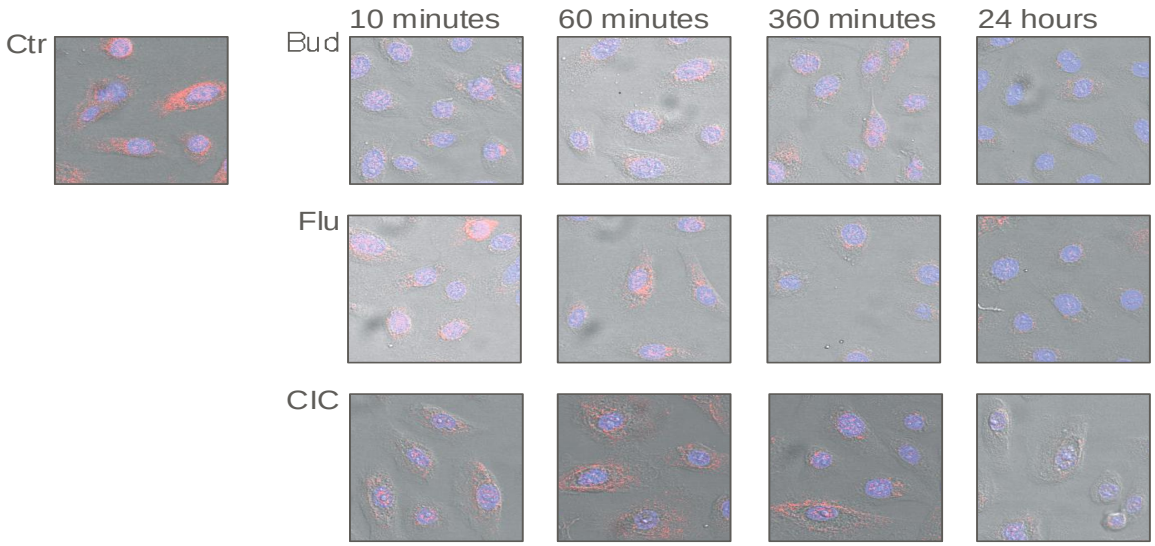


Figure 3*: Immunohistochemistry of primary human bronchial epithelial cells detecting the GR. Cells were treated 24, 6, 1 hour or 10 minutes before harvesting as indicated. Untreated cells served as control. Red colour depicts the GR, the nucleus was stained with To-RPO3, depicted in blue. Bud – budesonide, Flu – fluticasone, CIC – ciclesonide, all at* 10^{-8} M

Effects of steroid treatment on bronchial smooth muscle cell proliferation

Bronchial smooth muscle cells were treated with various glucocorticoids to protect against platelet-derived growth factor- (PDGF-) induced cell proliferation or glucocorticoid pre-treatment of resting cells to inhibit PDGF-induced cell proliferation, as two methods to investigate the steroid anti-proliferative ability in bronchial smooth muscle cell proliferation. We were measuring [^{3}H]-thymidine incorporation as a marker for DNA replication.

The results showed that RU486 had no effect on cell proliferation between 10^{-11} M and 10^{-7} M. Dexamethasone at low concentrations (10^{-11} M or 10^{-10} M) increased cell proliferation before dexamethasone became antiproliferative at 10^{-7} M. Both, budesonide and fluticasone were more potent in the inhibition of cell proliferation, leading to significant inhibition of dell proliferation between 10^{-10} M (fluticasone) and 10^{-9} M (budesonide). In contrast, ciclesonide

was not able to inhibit cell proliferation unless at concentrations > 10^{-8} M. To indicate glucocorticoid dependency of the inhibition of cell proliferation, RU486 (10^{-7} M) inhibited dexamethasone induced cell proliferation (figure 5).

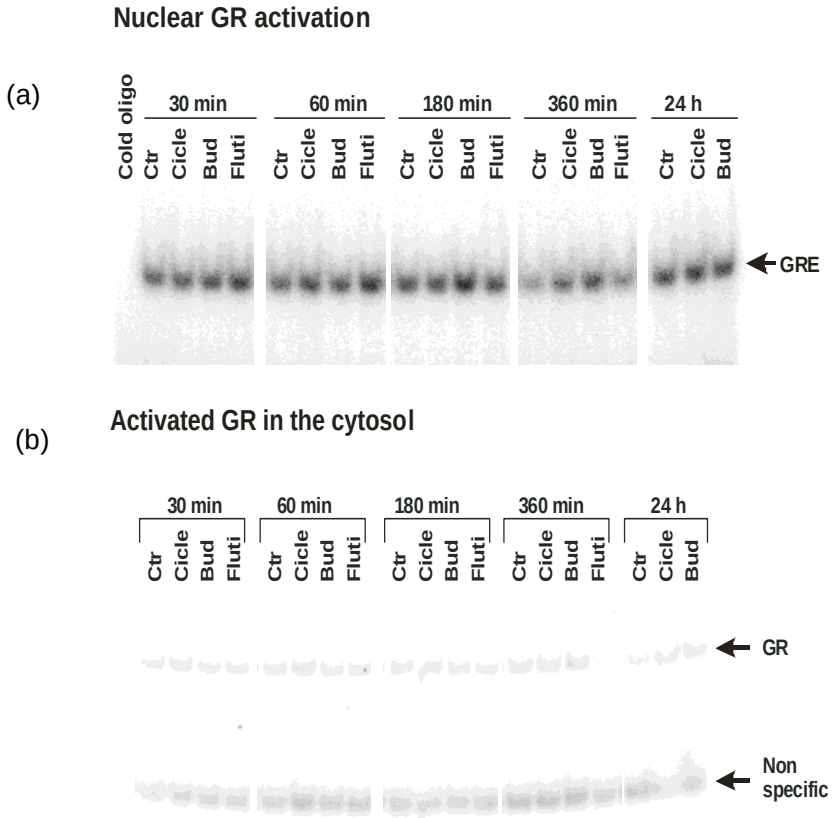


Figure 4*: Electrophoretic mobility shift assay (EMSA) using nuclear or cytosolic extracts of bronchial smooth muscle cells after treatment. Time points are indicated in the first line. In contrast to Western blot, EMSA is able to distinguish between activated GR and non-activated GR. Thus, nuclear GR signal (a) is 10-100 fold stronger than in cytosolic extracts (b).*

To investigate whether bronchial smooth muscle cells are able to convert ciclesonide into its active metabolite and how washing the cells influence the anti-proliferative properties of steroids. Cells were incubated for 12 hours with steroids removed thereafter. Subsequently, cell proliferation was stimulated using 5 % FCS. At high concentrations, ciclesonide and ciclesonide metabolite-1 appeared to be more potent than dexamethasone, budesonide or fluticasone. There was no significant difference between ciclesonide and its active metabolite. This indicates that bronchial smooth muscle cells were able to convert ciclesonide into its active form within the 12 hours of pre-incubation, thus ciclesonide treated cells were growth

arrested when cells became FCS-stimulated. Interestingly, fluticasone and budesonide could be washed away much more easily than ciclesonide or ciclesonide metabolite-1. (Figure 6). Bronchial smooth muscle cells were able to convert ciclesonide into its active form within the 12 hours of pre-incubation. Ciclesonide and ciclesonide metabolite-1 seem to persist longer within the cell than fluticasone and budesonide or are stronger bound to cell culture plastic or lipophilic cellular compartments.

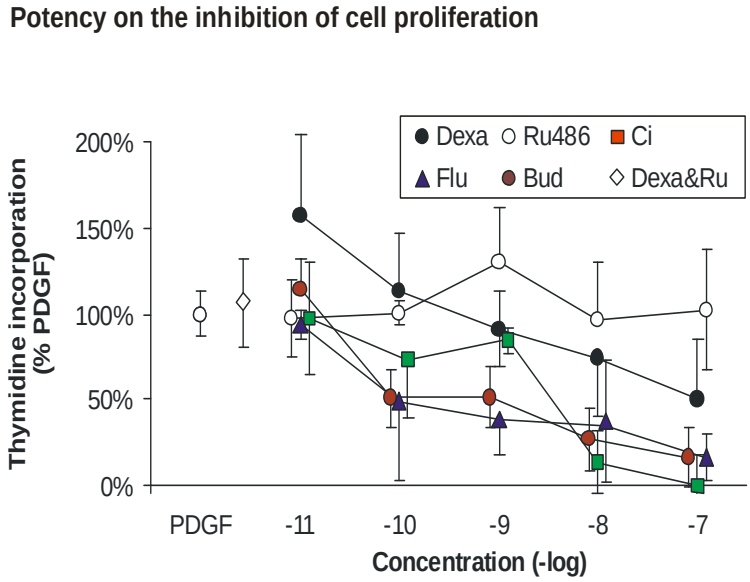


Figure 5: *Thymidine incorporation of BSMCs after removal of the respective steroids. Cells were treated with either dexamethasone (Dexa), ciclesonide (Ci), budesonide (Bud) or fluticasone (Flu) at the concentrations indicated. RU486 (mifepristone, an antiglucocorticoid) was used as non-GR activating steroid (10^{-7} M). Dexa&Ru indicates combined treatment of Ru486 (10^{-6} M) and dexamethasone (10^{-7} M). To avoid esterase contamination, cells were stimulated with platelet derived growth factor BB (PDGF), a cytokine known to be a strong inducer of BSMC proliferation (Carlin et al., 1999).*

At high concentrations, ciclesonide and ciclesonide metabolite I appeared to be more potent than dexamethasone, budesonide or fluticasone. At 10-8 M, budesonide and fluticasone were not active at all. There was no significant difference between ciclesonide and its active metabolite. This indicates that BSMC were able to convert ciclesonide into its active form within the 12 hours of preincubation, thus ciclesonide treated cells were growth arrested when cells became FCS-stimulated. Interestingly, fluticasone and budesonide could be washed away much more easily than ciclesonide or ciclesonide metabolite-1. To investigate, whether epithelial cells may affect the action of ciclesonide on the proliferation of airway smooth muscle cells, cells were treated in the presence of A549, a human airway epithelial tumour cell line. Thymidine incorporation was reduced by 20 % in ciclesonide treated cells without

epithelial cells. In the presence of epithelial cells proliferation was reduced by more than 50 %. Co-culture of airway smooth muscle cells with epithelial cells seems to speed up ciclesonide metabolism with subsequent inhibition of airway smooth muscle cell proliferation (figure 7).

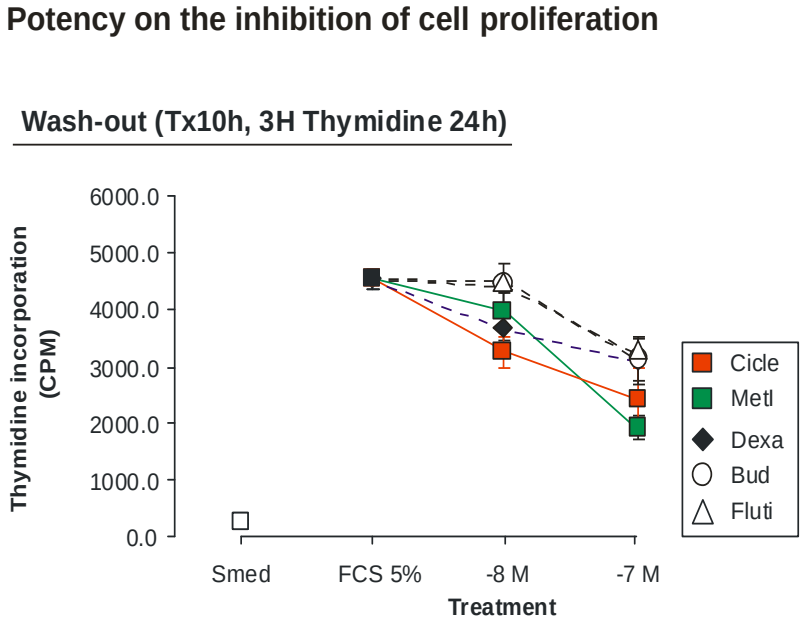


Figure 6: *Thymidine incorporation of BSMCs after removal of the respective steroids. Cells were treated with either ciclesonide (Cicle), ciclesonide metabolite-1 (MetI), dexamethasone (Dexa), budesonide (Bud) or fluticasone (Fluti) at the concentrations indicated. After 12 hours, medium containing the drugs was removed and cells were stimulated with 5 % FCS for 24 hours in presence of ^{3}H-thymidine. Thus in contrast to the experiments shown in figure 4 and 6, this experiment lasted for 36 instead of 24 hours.*

4.2.5 Discussion

Inhaled glucocorticoids are the corner stone of the treatment of mild to severe asthma. After activated glucocorticoid receptors bind to specific DNA binding site, the GRE thereby changing gene transcription of pro-inflammatory cytokines. Besides recruitment of inflammatory cells, these cytokines may lead to hyper-proliferation of bronchial smooth muscle cells observed in asthma, In contrast to their binding affinity, glucocorticoid potency in glucocorticoid receptor activation has not been fully investigated for modern glucocorticoids. We have compared the novel glucocorticoid ciclesonide with the most common inhaled glucocorticoids budesonide and fluticasone on glucocorticoid receptor activation in bronchial smooth muscle cells and their potency on the inhabitation of smooth muscle cell proliferation. To investigate, whether ciclesonide provides longer lasting

glucocorticoid receptor activation, therefore we investigated the effects of different steroids on kinetic of GR translocation and proliferation in human primary bronchial smooth muscle cells.

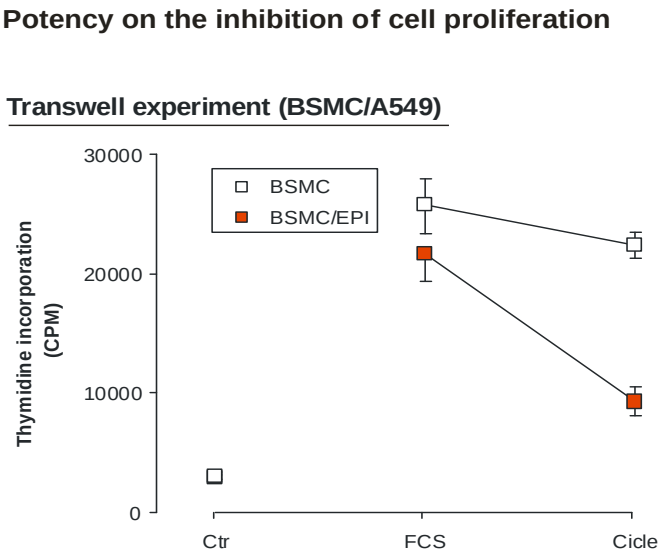


Figure 7: *Thymidine incorporation of BSMCs in presence of epithelial cells (transwell experiment) BSMCs were subconfluently plated and transwells were installed. Cells were stimulated using heat-inactivated 5 % FCS in presence or absence of ciclesonide (Cicle; 10^{-8} M). After 24 hours, transwells were removed and [3H] thymidine was added to cell culture at 5 µCi/ml for further 4 hours. Ciclesonide was more potent in presence of A549 than without.*

We showed that similar to fluticasone and budesonide, ciclesonide or ciclesonide metabolite-1 were in activating and translocation of the GR. The effect of ciclesonide was greatly retarded, when compared to ciclesonide metabolite-1, this obviously affected cell proliferation, where ciclesonide appeared to be less potent. When cells have been pretreated for 12 hours with either drug, we could not observe a difference between ciclesonide and its metabolite. This indicates that bronchial smooth muscle cells are slow metabolisers of ciclesonide. In presence of epithelial cells, ciclesonide was a more potent inhibitor of serum induced cell proliferation, supporting that epithelial cells, present in the human airway, are augmenting ciclesonide action on cell growth of bronchial smooth muscle cells. In contrast to fluticasone or budesonide, translocation of the GR was slower in bronchial smooth muscle cells treated with ciclesonide. Interestingly, GR translocation of ciclesonide metabolite-1 had a similar kinetic to fluticasone or budesonide. Same as all glucocorticoids were.

We conclude that bronchial smooth muscle cells are slow in metabolising ciclesonide and thus lead to retarded release of the active metabolite-1. To compare the speed of ciclesonide

activation in airway smooth muscle cells and epithelial cells, airway smooth muscle cell proliferation was investigated in the absence or presence of an airway epithelial cell line (A549). Co-culture with epithelial cells increased inhibition of airway smooth muscle cell proliferation. This may be interpreted that the combination of airway smooth muscle cells with A549 cells seems to speed up ciclesonide metabolism with subsequent inhibition of cell proliferation. This result needed to be confirmed in the primary human epithelial cells. We are not sure that primary human epithelial cells have same esterase and activity of A549 cells. Physiological relevance of the activation of the glucocorticoid receptor was assessed using cell proliferation assay according to two different experimental setups. In the first setup, we were measuring the effect of different glucocorticoids on platelet-derived growth factor-(PDGF-BB) stimulated cell proliferation, where ciclesonide showed similar efficacy in inhibition of airway smooth muscle cell proliferation compared to fluticasone and budesonide. But fluticasone (significant inhibition beginning at 10^{-10} M) and budesonide (significant inhibition beginning at 10^{-9} M) were more potent than ciclesonide (significant inhibition at concentrations of 10^{-8} M and 10^{-7} M). In the second setup of proliferation experiments, the steroids were incubated for 12 hours with airway smooth muscle cells; subsequently the supernatant was removed before growth stimulation without addition of glucocorticoids. Under these conditions, ciclesonide and des-CIC had comparable effects and were more potent and effective than budesonide or fluticasone. This implies, that ciclesonide can be activated by airway smooth muscle cells within 12 hours and persists longer within the cell than fluticasone and budesonide.

Our data might have some practical implications for the clinical use of ciclesonide. As we have shown that ciclesonide needs the activation by epithelial cells its action might be significantly delayed in diseases where the layer of epithelial cells is severely damaged, such as in COPD. Clinical data suggetes that ciclesonide is beneficial in mild to moderate asthmatics, but we may doubt its effect in patients with severe asthma, again due to a significant damage of the epithelial cell layer. When we compare the action of ciclesonide as a long acting steroid to that of mometasone, our data implies that mometasone is the better of both drugs budesonide and Dexametasone. This might be further supported by the observation that mometasone did not induce the depletion of the glucocorticoid receptor at 24 hours, while the ciclesonide metabolite-1 did, and therefore is not different form classical short acting steroids.

In conclusion the above data indicate that the molecular mechanism underlying the long lasting effect of ciclesonide is not different from that of classical glucocorticoids but depends on its activation by epithelial cells.

This data has been expanded and that it is currently prepared for publication.

CHAPTER 5

KEY FINDINGS

Key findings

My thesis aimed to determine the effect of inflammation on the expression and function of the GR in the human lung using a cell culture model with primary mesenchymal cell types including connective tissue fibroblasts and bronchial smooth muscle cells of non-diseased human lungs. The thesis also aimed to characterize the mode of action of two novel long acting steroids, mometasone and ciclesonide, and to compare their mode of action on cell biology. The highlights of these experiments summarized are:

1. Cell density and inflammation alter the distribution and function of the GR.
2. Mometasone does not induce tachyphylaxis and has a preferable effect on airway remodelling.
3. The long lasting effect of ciclesonide depends on its metabolisation by epithelial cells
4. The activation of ciclosonide metabolite is not different from that of classical glucocorticoids.

APPENDIX

MATERIALS AND METHODS

Primary human lung cell cultures

The primary human lung cell cultures were established from macroscopically normal parts of peripheral lung tissue obtained from patients undergoing partial lung resection as part of a lung cancer therapy. This protocol was approved by the Ethical committee of the Faculty of Medicine, University Hospital Basel (#M75/97). Tissues were collected from the Department of Thoracic Surgery. All patients gave written and informed consent. Primary human lung fibroblast cultures are well established in our laboratory and their phenotype was previously characterized and described by our group (Roth et al., 1996;Papakonstantinou et al., 2000;Papakonstantinou et al., 2003)

Briefly, fibroblasts were established from sterile lung tissue samples stored at 4℃ in sterile PBS (Cambrex Bio Science, Verviers, Belgium). Tissue samples were cut into small pieces of 1-2 mm3 and 16-20 pieces were placed into 75 cm2 flasks pre-wetted with 5 ml of RPMI 1640 (2.1 mM glutamine and 25 mM Hepes; Cambrex) supplemented with 10% FCS (heat inactivated for 15 min at 65℃; Gibco BRL Invitrogen, Basel, Switzerland) and 1% MEM-Essential minimum vitamins (Cambrex). Cultures were performed without antibiotics and were kept at 37℃ in a humidified incubator containing 5% CO2. Epithelial-like cells started to grow after few days followed by spreading of spindle shape-like fibroblasts from the tissue pieces. Medium was changed twice a week until fibroblasts reached confluence around the tissue pieces. Then, cells were washed three times with PBS and trypsinized [0.125% trypsin (Biochrom AG, Berlin, Germany) and 0.01% EDTA in PBS] for 3 min at 37℃. For the first passage, cells were re-suspended in 10% FCS medium in the same flask to avoid any cell loss and medium was changed the following day. The next passages were performed at a 1:3 ratio.

Fibroblasts displayed a typical spindle-shaped morphology, stained positive for fibronectin and laminin but were negative for von Willebrand factor, Factor VIII, cytokeratin, and smooth muscle actin. Fibroblasts between passages 3 and 6 were used for all experiments.

Primary cultures of human BSMC were grown from airway muscle bundles obtained from probands. Cells were grown in RPMI 1640 containing 10 % foetal calf serum, and 20 mM HEPES. All experiments were performed between passages 3 – 6. Prior to experiments, cells were serum starved for 48 hours using RPMI-1604 containing 0.1 % FCS. No antibiotics were used at any time. (Johnson et al., 2001;Johnson, 2001).

Cell stimulation and drug treatment Serum starvation

Prior to stimulation and treatment, cells were growth-arrested by serum starvation for 24 h in low serum RPMI 1640 medium supplemented with 0.1% FCS. Medium was exchanged after 12 and 24 h to avoid auto-stimulation of the cells.

dexamethasone from Calbiochem, Lucerne, Switzerland, budesonide from AstraZeneca, Lund, Sweden and ciclesonide and ciclesonide metabolite I from Altana, Germany. Experiments at concentrations ranging from 10^{-6} to 10^{-10} M for 24 or 48 h under both non-inflammatory and inflammatory conditions. Drugs were dissolved in DMSO, which final concentration (< 0.01% DMSO in the culture medium) had no effect on all measured parameters.

Total proteins concentration

Total proteins concentration in conditioned media was measured using the Bradford protein assay (Bio-Rad Laboratories GmbH, Munich, Germany). The standard curve was established using bovine serum albumin (0-500 ng/ml) and the absorbance was measured at 595 nm with a microplate reader (SpectraMax 190, Molecular Devices Corporation, Sunnyvale, USA).

RNA extraction and reverse transcription

Cells were plated in 100 x 20 mm dishes, grown until confluence and serum-deprived for 24 h prior to stimulation and/or drug treatment. After incubation, cells were washed twice with ice- cold PBS, scrapped and cell pellets were collected by centrifugation at 900 x *g* for 10 min. Total RNA was isolated using RNeasy Mini kit (Qiagen, Basel, Switzerland) and RNA concentration and purity were measured by spectrophotometry (NanoDrop Technologies Inc., Wilmington, DE, USA). The A260/A280 ratio was used as an indicator of purity with optimal values ranging between 1.8 and 2.0 RNA samples were stored at -70 ℃ until analysis. First strand cDNA was synthesized from 1 µg of total RNA using M-MLV

Reverse Transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA).

Polymerase chain reaction

cDNA was amplified using *Taq* DNA polymerase (Promega). PCR conditions consisted on 1) an initial denaturation at 95 ℃ for 2 min, 2) denaturation at 98 ℃ for 20 sec, primer annealing at 58 ℃ for 22 sec, and extension at 72 ℃ for 30 sec to 1 min for 20-40 cycles, and 3) a final extension at 72 ℃ for 5 to 10 min. The intensity of each band was analyzed by densitometry using the NIH Image J software and the relative mRNA expression of target gene was normalized to β2-microglobulin (β2-M). All primers were purchased from MWG-Biotech AG (Ebersberg, Germany). The sequence of GR primer: forward:5'-CACCCTCACTGGCTGTCGCTTCTC-3', reverse:5'TGACAAACGAAA GAGGAG ACCGCC-3', 23 cycles: denaturation: (98 ℃, 20 sec), annealing (58 ℃, 22 sec) and extension (72 ℃, 30 sec); p21 (primer set, R&D Systems Inc, Minneaplois, USA), 30 cycles: denaturation: (94 ℃, 45 sec), annealing (55 ℃, 45 sec) and extension (72 ℃, 45 sec); β2-microglobulin (β2-M): forward 5'-CTCGCGCTACTCTCTCTCTT TCT3',reverse:5'TTAAGTGGGATCGAGACATGTAAGC-3', 23 cycles: denaturation (98 ℃, 30 sec), annealing (60 ℃, 60 sec) and extension (72 ℃, 60 sec). PCR products were size fractionated by electrophoresis in a 1 % agarose gel and products were visualized by ethidium bromide.

Immuno-histochemistry:

Fibroblasts were seeded (10^4 cells/ml) in 24 well plates and serum deprived (24 hrs) before being stimulated with 10% FCS in the presence or absence of dexamethasone or mometasone fuorate (10^{-6} or 10^{-8}M) for 0, 6, 18, 24 or 48 hrs. Cells were washed with phosphate buffered saline (PBS; 3 x 5 min), fixated in ice-cold methanol:acidic acid (3:1, 15 min), and washed twice with PBS. Endogenous peroxidase was blocked (0.1% NaN3, 0.3% H_2O_2; 30 min, room temperature), followed by 2 washes with PBS, and unspecific antibody binding was blocked (5 % FCS, 0.5% Tween-20 in PBS; 1 hrs, room temperature). Then slides were incubated with a polyclonal rabbit anti-glucocorticoid receptor antibody (sc-1003, Santa Cruz Biotechnology, Santa Cruz, CA; 1:400 in blocking buffer' 4°C, overnight) before being washed twice with PBS and incubated with a horseradish-peroxidase-labelled goat anti-rabbit antibody (sc-2004, 1:1000 in blocking buffer, 1 hr, room temperature). Slides were then

washed with PBS (3 x 5 min) and the developing solution AEC Chromogen Kit (AEC101, Sigma, Saint louis, Missouri, USA) was added according to instructions until a red signal was obtained in the positive control. Slides were washed twice with deionized water and images were captured using Anlysis-D soft imaging system with Olympus IX50 microscope (objective 40 x). The same procedure was performed for $p21^{(Waf1/Cip1)}$ using a polyclonal mouse anti-$p21^{(Waf1/Cip1)}$ antibody (610234, BD Biosciences Pharmingen San Diego, CA, USA) diluted 1:300 in blocking buffer. The secondary antibody was a horseradish-peroxidase-labelled goat anti-mouse antibody (sc-2005; diluted 1:1000 in blocking buffer) incubating the slides for 1 hr at room temperature.

Nuclear and cytosolic extracts and immuno-blotting:

Cytosolic and nuclear proteins were prepared from 80 % confluent fibroblasts and the protein-concentration was measured according to Bradford's method (Biorad) as described earlier (19).Protein expression was determined in 10 µg of total protein that were size-fractionated in a denaturing 4-15 % SDS-PAGE (19). Proteins were transferred onto PVDF membrane (Millipore Corp., MA, USA) and transfer was controlled by Ponceau's staining. Membranes were incubated for 1 hr with blocking buffer (10 mM Tris, 150 mM NaCl, 0.05 % Tween 20, 5 % skimmed milk), followed by overnight incubation (4℃) with either a polyclonal anti-glucocorticoid receptor antibody, an antibody for C/EBP -α , or $p21^{(Waf1/Cip1)}$ (sc-8992, sc-61, sc-6246; all: Santa Cruz Biotech). Unbound antibody was washed of (2 x blocking buffer) and membranes were incubated (30 min) with a 2^{nd} horseradish-peroxidase-labelled anti-rabbit antibody (Chemicon). Protein bands were detected by enhanced chemiluminescence (ECL, Pierce, IL, USA) exposed to X-ray films. Optical density of protein-signals was evaluated by using AIDA 2.0 and ImageJ (19).

Statistical analysis

Results of protein activation kinetics were compared by paired ANOVA. The effect of signal protein inhibitors and siRNAs were compared by paired two-tailed student’s t-test. Results were considered to be significantly different when the probability (p) was < 0.05.

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